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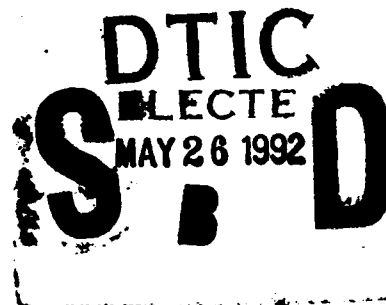
**TITLE: PATHOGENESIS OF DIC IN STRAIN 13 GUINEA PIGS  
INFECTED WITH PICHINDE VIRUS**

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## INTRODUCTION

Arenaviruses include a group of viruses that have an RNA genome, are inactivated by lipid solvents, and share immunologic epitopes. Lymphocytic choriomeningitis (LCM) virus, the first recognized member of this group, is considered the prototype of arenaviruses and possesses a unique morphology of oval or pleomorphic shape, an electron-dense membrane with spikes, and a number of inclusion-like, dense particles that give the virion a feature of having been sand-sprinkled (arenosus) (1).

Arenaviruses include four human pathogens, of which three develop hemorrhagic fever: LCM, Lassa, Junin and Machupo viruses (2). LCM virus has a worldwide distribution but rarely causes severe disease in man. The other three cause viral hemorrhagic fevers in West Africa (Lassa fever by Lassa virus) and South America (Argentine hemorrhagic fever by Junin virus; Bolivian hemorrhagic fever by Machupo virus). The arenaviral hemorrhagic fevers (AVHF) have several clinical features in common: 1) a viral reservoir in wild rodents; 2) a hemorrhagic diathesis; 3) an acute encephalopathy; and 4) a 10-20% mortality rate in untreated hospitalized patients (2). The risk of importing AVHF into the United States is small but real and, with increasing intercontinental travel, it continues to be a worldwide health concern among public health officials (3).

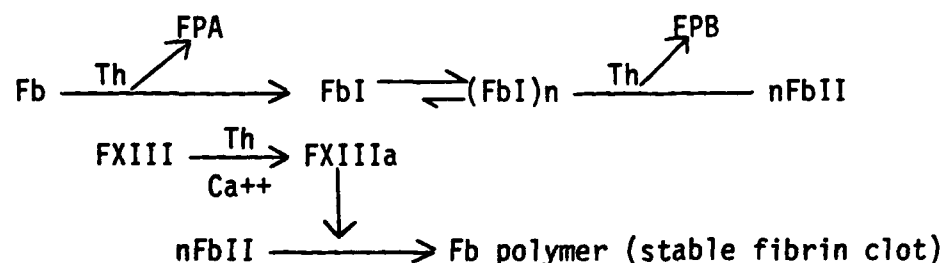
In 1981, an animal model for studying the pathogenesis of arenavirus infection was developed at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID). Pichinde virus, a Colombian arenavirus of the Tacaribe group, which does not cause disease in humans, was adapted to produce a lethal infection in strain 13 guinea pigs (4). The virological and pathological features of experimental Pichinde infection in inbred guinea pigs are remarkably similar to those described for Lassa virus infection in humans.

Utilizing this animal model, the pathogenesis of Lassa fever and other AVHFs can be studied outside the maximum containment (P-4) laboratory.

Cosgriff et al. (5) reported that significant coagulation disorders were associated with the experimental infection of stain 13 guinea pigs with Pichinde virus. Infected animals developed decreased activity of multiple coagulation factors, decreased antithrombin III levels, elevated serum levels of fibrinogen-fibrin degradation products (FDP), impaired platelet function, and thrombocytopenia. The symptomatology described by these workers is suggestive of disseminated intravascular coagulation (DIC) (6). However, reduced plasma levels of multiple coagulation factors could be the result of either increased consumption or impaired synthesis. Increased consumption occurs in DIC, whereas impaired synthesis is the probable consequence of liver injury. The liver is the known or presumed site of synthesis for most coagulation factors (7).

#### SPECIFIC AIMS

Thrombin, which is generated after the activation of the coagulation pathway, will liberate fibrinopeptides A and B (FPA and FPB) from fibrinogen to yield fibrin monomers, which polymerize to form fibrin polymers. This polymer, following factor XIII-mediated crosslinking, becomes a stable fibrin clot. The multi-step process of fibrinogen-fibrin conversion is shown below:



A sensitive method of specifically measuring one of these peptides should prove useful in studying coagulation in vivo and thrombin action in vitro. We

report here: 1) the development of a radioimmunoassay (RIA) of guinea pig fibrinopeptide A (gFPA) and its application to measure the thrombin activity in inbred guinea pigs during the course of infection with Pichinde virus; 2) the validation of the gFPA-RIA by determining the quantity of gFPA released from thrombin-degraded guinea pig fibrinogen; 3) the determination of immunological cross-reactivity with fragment D or E exhibited by anti-gFPA antibody.

#### MATERIALS AND METHODS

Viral stocks: The Pichinde virus used in these studies was kindly provided by Dr. David Gangemy, Department of Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC. The adapted Pichinde virus was produced by serial passage (13X) of virus strain AN4763 in strain 13 guinea pigs, as described by Jahrling et al. (4). The titer of this virus was ca.  $7\log_{10}$  plaque forming units (PFU) on Vero cell culture monolayers.

Infection of guinea pigs and blood sampling: Male strain 13 guinea pigs, 700-850 g, were purchased from Crest-Caviary, Raymond, CA. Animals were housed individually in plastic cages, with control and infected animals in separate rooms. Test animals were inoculated intramuscularly in the thigh with virus suspensions containing  $7\log_{10}$  PFU of pichinde virus diluted in RPMI 1640 (Cell Culture Laboratories, Cleveland, OH). Virus suspensions consisted of a 10% suspension of the spleen homogenate of infected inbred guinea pigs in RPMI 1640 containing 2% fetal calf serum and 2% penicillin and streptomycin.

Guinea pigs were sedated with a mixture of 35 mg/kg body weight of ketamine HCl and 5 mg/kg of xylazine, and bled by cardiac puncture. Blood for the gFPA-RIA was drawn from a single puncture of 5.4 ml sample, collected into siliconized Vacutainer tubes. Vacutainer tubes contained 0.6 ml of 0.1 M sodium citrate containing 2500 kallikrein inhibitor units of aprotinin (Sigma Chemical Co., St. Louis, MO) and 1000 units of heparin (Upjohn Co., Kalamazoo,



MI). Sample blood was mixed with the anticoagulant immediately, placed in melting ice, and centrifuged at 2600 rpm for 20 min. The plasma was centrifuged at 10,000 rpm for an additional 20 min at 4°C, and "snap-frozen" in ethanol-dry ice before being stored at -70°C.

Plasma sample processing: The procedure of processing guinea pig plasma for the gFPA-RIA was a simplified procedure derived from the method of Nossel et al. (8) for processing human plasma. One ml of plasma was mixed with 3 ml of 95% ethanol and allowed to stand at room temperature for 30 min with occasional stirring. The precipitate was removed by centrifugation at 5000 rpm for 20 min. The supernatant was concentrated in an RCF Confilt hollow fiber apparatus (Spectrum, Houston, TX) by centrifuging at 2500 rpm for 30 min. The ethanol-water mixture in the filtrate was removed under vacuum by evaporation in a Speed Vac (Savant Instruments, Farmingdale, NY). This machine allows the solution to be subjected to a vacuum while suppressing boiling by centrifugation. The residue remaining after vacuum drying was re-constituted in 1 ml of diluent phosphate-buffered saline (PBS), pH 7.4., containing 0.1% bovine serum albumin (BSA), and was used directly in the gFPA-RIA.

Synthesis of guinea pig fibrinopeptide A and its analogs: The amino acid sequence of gFPA has been determined by Blomback et al. (9) as follows:

Thr-Asp-Thr-Glu-Phe-Glu-Ala-Ala-Gly-Gly-Gly-Val-Arg

or TDTEFEAAGGGVR with amino acid residues expressed in the single letter code (10). Guinea pig FPA lacks tyrosine; in order to label it with radioactive iodine, tyrosine was inserted into gFPA at two different positions. Tyrosine was introduced into gFPA in place of phenylalanine-5 or in lieu of valine-12.

The Analytical Services Facility of the University of Tennessee/Knoxville performed the synthesis of gFPA and its two analogs, viz., 5-Tyr-gFPA

(TDTEYDAAGGGVR) and 12-Tyr-gFPA (TDTEFEAAGGGYR). Briefly, the synthesis of the three analogous peptides was accomplished by means of ter-butyloxycarbonyl (t-BOC) amino acid conjugated to chloromethyl polymer in a Biosearch SAM II automatic peptide synthesizer. Removal of t-BOC protecting groups and couplings gradually built up the peptide chain. Finally, peptides were cleaved from the resin by incubation for 1 hr at 0°C in anhydrous hydrofluoric acid. The peptides were solubilized in 50% acetic acid and chromatographed on a Bio-Rad P-2 column. The peptides were then subjected to high pressure liquid chromatography on a Vydac C-17 reverse-phase column using 0.1% trifluoroacetic acid as a modifier and acetonitrile as the eluent. The major peak was collected. Amino acid analysis confirmed these peaks to contain the target peptides.

Immunogens and antisera: Guinea pig FPA was conjugated to BSA by two different methods of chemical coupling. Synthetic gFPA was coupled to BSA via glutaraldehyde (12). A solution was made consisting of 7 mg BSA (crystallized, Sigma Chemical Co., St. Louis, MO) and 2 mg gFPA dissolved in 1 ml of 0.05 M PBS, pH 7.2. One-half ml of 0.025 M glutaraldehyde was added dropwise in 5 min to the reaction mixture with constant stirring. The coupling reaction was continued by gentle agitation at room temperature for 20 min. The reaction was terminated by dialysis against 0.05 M PBS overnight at 4°C.

Alternatively, synthetic gFPA was coupled to BSA by means of 1-cyclohexyl-3-(2-morpholinylethyl)carbodiimide HCl (morpho CDI) (13). Ten mg of BSA and 14 mg of gFPA were dissolved together in 1 ml of water. To this mixture, was added drop by drop 1 mg aqueous solution of 75 mg morpho CDI (Aldrich Chemical Co., Milwaukee, WI). The conjugate remained in solution during the dropwise addition of morpho CDI (5 min), but precipitated thereafter. The reaction was continued with gentle agitation at room

temperature for 30min. The reaction was terminated by dialysis against 0.05 M PBS overnight at 4°C.

By the glutaraldehyde method, gFPA was presumably conjugated to BSA by the N-terminal end only. The gFPA-BSA conjugate by the carbodiimide method are thought to be covalently linked by either the free  $\text{NH}_2$  or  $\text{CO}_2\text{H}$  groups of the gFPA molecules. Antibodies to gFPA were elicited by immunizing New Zealand white rabbits with the mixture of gFPA-BSA conjugate and Freund's adjuvant as described previously (14), except that the route of immunogen administration was altered slightly: The adjuvant mixture was administered subcutaneously in the back and intramuscularly in the hind legs. Fur was shaved from the back and proximal limbs of the rabbits, and the emulsion was injected subcutaneously in the back and the remainder intramuscularly in the two hind legs.

The antisera derived from three rabbits immunized with gFPA-BSA conjugate prepared by the glutaraldehyde method were designated as R125, R126 and R127 antisera. Antisera derived from three rabbits injected with gFPA-BSA conjugates prepared by the carbodiimide method were termed R128, R129 and R130 antisera.

Radioimmunoassay: Tyrosinated gFPA was radiolabeled with [ $^{125}\text{I}$ ]NaI by the iodogen method of Fraker and Speck (15), with a modification introduced by Chen et al. (16). The iodogen (1,3,4,6-tetrachloro-3a,6a-diphenyl glycoluril) was purchased from Pierce, Rockford, IL. To increase the sensitivity of the gFPA-RIA, the assays were performed by pre-incubating the antibody with a test sample before the radiolabeled ligand was added (delayed addition of the tracer) (17). The RIA procedure was carried out in duplicate and included control tubes with  $^{125}\text{I}$ -tyrosinated gFPA but without anti-gFPA-BSA serum (co-precipitation). The following reagents were added in sequence to polystyrene

test tubes (5 ml capacity): (a) serial dilutions of standard gFPA or test sample made up to a total volume of 100  $\mu$ l in diluent PBS, pH 7.4, containing 0.1% rabbit serum albumin (RSA); (b) 100  $\mu$ l of R125 antiserum suitably diluted with diluent PBS; and (c) 500  $\mu$ l of diluent PBS. After overnight incubation at 4°C for 18 hr, 100  $\mu$ l of  $^{125}$ I-5-Tyr-gFPA or  $^{125}$ I-12-Tyr-gFPA, which had been diluted with diluent PBS to give ca. 30,000 cpm, was added to the mixture, which was then incubated at 4°C for an additional 24 hr. The incubation volume was 700  $\mu$ l. Antibody-bound and free fractions were separated by the addition of 100  $\mu$ l of goat anti-rabbit gamma globulin (GARGG, 20% concentrated; Antibodies, Inc., Davis, CA). After waiting for 15 min, 200  $\mu$ l of polyethylene glycol (PEG) solution (7 g PEG in 14 ml diluent PBS) was added, and the reaction mixture incubated at room temperature for 1 hr more. The precipitates were centrifuged at 5000 rpm for 30 min and counted for 2 min in a Searle model 1185 gamma counter. Plasma gFPA immunoreactivities were estimated by using a graphic extrapolation of the plot for logit ( $B_i/B_o \times 100$ ) vs. gFPA concentration.

Thrombin degradation of guinea pig fibrinogen: Each experiment was performed using guinea pig fibrinogen (Sigma Chemical Co.) at a final concentration of 0.45 mg/ml in 0.1 M NaCl buffered with 0.05 M Tris-HCl at pH 7.4. 1.8 ml aliquots of fibrinogen at 0.5 mg/ml were incubated at 37°C with 200  $\mu$ l (0.2 NIH unit/ml) of human thrombin (Enzyme Research Laboratories, South Bend, IN) for intervals ranging from 5 to 60 min. Control samples of fibrinogen were incubated with Tris-saline buffer in place of enzyme at 37°C for 0 and 60 min. The reaction was stopped and the fibrinogen precipitated by the addition of 2 ml ethanol to each sample. The fibrinogen was deposited by centrifuging at 7,000 rpm at 4°C for 10 min. The top 2 ml of supernatant solution was transferred to a fresh tube and recentrifuged under the same conditions. Of this,

the top 1 ml of supernatant solution was pipetted into an RCF Confilt hollow fiber apparatus (Spectrum, Houston, TX) and centrifuged at 2,400 rpm for 25 min. The ethanol-water mixture in the filtrate was removed under vacuum by evaporation in a Speed Vac. The residue remaining after vacuum drying was reconstituted in 1 ml of diluent PBS, pH 7.4, containing 0.1% BSA, and was used directly in the gFPA-RIA.

Fragments D and E: Twenty mg of guinea pig fibrinogen (Sigma Chemical Co.) was mixed with 1.26 caseinolytic units of human plasminogen (AB Kabi, Stockholm, Sweden). The plasminogen was activated by adding 11.28 Plough units of urokinase (Leo Pharmaceutical Co., Ballerup, Denmark). Digestion at 37°C was terminated after 6 hr by adding aprotinin (Sigma Chemical Co.) to a final concentration of 200 kallikrein inhibitor (KI) U/ml. The degree of digestion of the fibrinogen digest was determined by immunoelectrophoresis with anti-fibrinogen serum, dependent on the presence (or absence) of the intermediate fragments X and Y (18).

The 6-hr digest of fibrinogen was dialyzed against 0.0001 M dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) to remove small peptides and designated as the D:E complex. Separation of the complex into constituent D and E fragments was done by passage through DEAE-cellulose (DE52, Whatman Lab Sales, Hillsboro, OR) column and elution with a phosphate gradient (0.0001 M  $\text{Na}_2\text{HPO}_4$  to 0.5 M  $\text{Na}_2\text{HPO}_4$ ), as described by Nilehn (19).

## RESULTS

Antiserum specificity and assay sensitivity: The antisera, derived from three rabbits immunized with gFPA-BSA conjugates prepared by the glutaraldehyde method, showed a specificity toward gFPA. The specificity was revealed by the double antibody RIA employing  $^{125}\text{I}$ -labeled-5-Tyr-gFPA as a radioactive tracer and R125 rabbit anti-gFPA-BSA conjugate as a polyclonal antibody. Of

the three harvested antisera, R125 antiserum exhibited the greatest sensitivity toward gFPA. The working range for gFPA was between 1 ng/ml and 1  $\mu$ g/ml (Fig. 1). Guinea pig fibrinogen inhibited the antigen-antibody reaction between  $^{125}\text{I}$ -5-Tyr-gFPA and R125 anti-gFPA-BSA antibody from 1.4  $\mu$ g/ml to 4  $\mu$ g/ml. Using  $^{125}\text{I}$ -12-Tyr-gFPA as a radioactive tracer, R125 antiserum also showed a similar specificity toward gFPA. The working range for gFPA was between 6 ng/ml and 40 ng/ml (Fig. 2). Guinea pig fibrinogen also cross-reacted with  $^{125}\text{I}$ -12-Tyr-gFPA from 4.5  $\mu$ g/ml to 10  $\mu$ g/ml against R125 anti-gFPA-BSA antibody.

The antisera, derived from three rabbits immunized with gFPA-BSA conjugate prepared by the carboimide method, likewise demonstrated a specificity toward gFPA. Of the three different antisera, R130 antiserum showed the greatest sensitivity toward gFPA in the double antibody RIA using  $^{125}\text{I}$ -5-Tyr-gFPA as a tracer. The working range for gFPA was between 20 ng/ml and 4  $\mu$ g/ml (Fig. 3). Guinea pig fibrinogen inhibited the RIA between  $^{125}\text{I}$ -12-Tyr-gFPA and R130 anti-gFPA-BSA antibody from 1  $\mu$ g/ml to 100  $\mu$ g/ml. Employing  $^{125}\text{I}$ -12-Tyr-gFPA as a tracer, R130 antiserum also showed a similar specificity toward gFPA. The working range for gFPA was between 2 ng/ml and 4  $\mu$ g/ml (Fig. 4). Guinea pig fibrinogen also cross-reacted with  $^{125}\text{I}$ -12-Tyr-gFPA from 1  $\mu$ g/ml to 100  $\mu$ g/ml against R130 anti-gFPA-BSA antibody.

Processing of plasma: Guinea pig FPA measurements in unextracted plasma were unsatisfactory because fibrinogen cross-reacted with the anti-gFPA-BSA antibody. Plasma sample processing was thus required to remove fibrinogen and FDP, which might contain FPA. Basically, three different procedures of sample plasma processing have been tested in our laboratory: 1) bentonite adsorption (20); 2) polyethylene glycol and heat precipitation (21), and 3) ethanol precipitation and dialysis (8). The first two procedures were not able to

completely remove immunologically cross-reacting material from some of the normal guinea pig plasmas. The final and most satisfactory procedure arrived at in our hands was to introduce the process of dialysis in addition to precipitating fibrinogen in 75% (v/v) ethanol-plasma mixture (22). This step was accomplished by centrifuging the supernatant (after ethanol precipitation) using a hollow fiber apparatus (see Materials and Methods section).

Normal plasma gFPA immunoreactivities: It was necessary to prevent in vitro generation of gFPA activity, and it was found that the lowest gFPA levels were obtained in normal guinea pig plasmas when a mixture of heparin and aprotinin was added to the blood (see Materials and Methods section). Table I lists the gFPA immunoreactivities determined by RIA after the ethanol precipitation-dialysis treatment of the plasmas from wild-type guinea pigs (Hartley).

TABLE I

<u>Plasma gFPA Levels in Healthy Hartley (Outbred) Guinea Pigs</u>			
GP1	14.0 ng/ml	GP9	6.56
	(10.6 pmoles/ml)		(4.95)
GP11	2.38	GP15	3.30
	(1.80)		(2.49)

Thrombin treatment of guinea pig fibrinogen: Incubation of guinea pig fibrinogen with human thrombin resulted in the cleavage of a segment of the N-terminal end of the A( $\alpha$ )-chain, viz., gFPA. The validation experiment for the gFPA-RIA was carried out by determining the amount of gFPA released from thrombin-degraded guinea pig fibrinogen. In the experiment, 80  $\mu$ mole/ml of ethylenediamine tetraacetate (EDTA) was added to the digestion mixture to stabilize the gFPA released by thrombin (23). After ethanol precipitation and

centrifugation, dialysates of enzymatically altered fibrinogen were assayed for gFPA (see Materials and Methods section for experimental details).

Fig. 5 and Fig. 6 show the yields of gFPA in the thrombin digest of guinea pig fibrinogen. Two different tracers were used in the RIAs; one consisted of  $^{125}\text{I}$ -5-Tyr-gFPA and R125 rabbit anti-gFPA-BSA serum in the assay system, and the other involved  $^{125}\text{I}$ -12-Tyr-gFPA and R125 antiserum. In both cases, a maximum amount of gFPA had been released after 20 min of incubation (Figs. 5 and 6). The peptide was found to be unstable at 37°C, despite the stabilizing effect of 80  $\mu\text{mole/ml}$  of EDTA, with considerable loss of immuno-reactivity after 40 and 60 min.  $^{125}\text{I}$ -12-Tyr-gFPA appeared to be less susceptible to proteolytic cleavage than  $^{125}\text{I}$ -5-Tyr-gFPA, since approximately 73 times in yield of gFPA (on molar basis) was detected by the RIA using  $^{125}\text{I}$ -5-Tyr-gFPA as the tracer.

Pichinde-infection of inbred guinea pigs: To produce "adapted Pichinde virus" for inoculation into strain 13 guinea pigs (4), the following procedure was adopted:

Colombian rice rat  $\xrightarrow{\text{Pichinde virus}}$  hamster brains

"Parent Pichinde"  $\longrightarrow$  Strain 13 GP  $\xrightarrow{13\text{X}}$  Spleen "adapted Pichinde"

The sequential passage (13X) of Pichinde virus in strain 13 guinea pigs resulted in the evolution of a virus uniformly lethal by day 10-12 for inbred guinea pigs of  $\sim 1$  kg. The protocol of Pichinde virus infection, blood collection and plasma processing for infected and control guinea pigs has been described in detail in the Materials and Methods section.

Tables II and III show the results of the gFPA-RIA done using two different tracers, i.e.,  $^{125}\text{I}$ -5-Tyr-gFPA and  $^{125}\text{I}$ -12-Tyr-gFPA. These two sets of



RIA data were derived from two separate experiments involving Pichinde virus infection of inbred guinea pigs.

TABLE II

Plasma of gFPA Levels of Infected and Control Strain 13 Guinea Pigs  
(<sup>125</sup>I-5-Tyr-gFPA as Tracer)

<u>CONTROL</u>	GP24	GP25	GP26	GP27
D0	2.05 ng/ml	0.53 ng/ml	0.63 ng/ml	1.42 ng/ml
D3	6.12	2.15	4.56	10.59
D7	7.46	1.85	4.31	1.13
D10	13.21	1.02	1.61	1.95
D12	3.64	0.80	2.01	0.97
<u>INFECTED</u>	GP28	GP29	GP30	GP31
D0	7.69 ng/ml	3.54 ng/ml	9.53 ng/ml	8.73 ng/ml
D3	13.08	4.36	5.11	807.12
D7	12.69	95.98	27.62	133.69
D10	32.25	40.26	3218.80	33.57
D12	35.97	181.71	Died	Died

(<sup>125</sup>I-12-Tyr-gFPA as Tracer)

<u>CONTROL</u>	GP24	GP25	GP26	GP27
D0	4.52 ng/ml	3.26 ng/ml	3.48 ng/ml	3.57 ng/ml
D3	6.83	3.28	17.85	7.15
D7	2.51	3.35	6.27	3.03
D10	1.86	1.45	6.39	3.13
D12	1.07	7.46	5.07	3.28
<u>INFECTED</u>	GP28	GP29	GP30	GP31
D0	38.06 ng/ml	4.50 ng/ml	6.87 ng/ml	6.56 ng/ml
D3	13.67	10.18	7.80	3471.88
D7	12.98	906.10	52.64	289.11
D10	80.99	499.32	5655.66	11.41
D12	401.46	6245.03	Died	Died

TABLE III

Plasma gFPA Levels of Infected and Control Strain 13 Guinea Pigs  
 ( $^{125}\text{I}$ -5-Tyr-gFPA as Tracer)

<u>CONTROL</u>	GP22	GP23	<u>INFECTED</u>	GP24A	GP26
D0	1.46 ng/ml	17.39 ng/ml	D0	14.39 ng/ml	29.98 ng/ml
D2	6.92	2.42	D2	6.61	94.22
D5	7.77	3.49	D5	11.67	70.18
D7	49.01	11.89	D7	2.10	494.79
D9	3.36	66.44	D9	Died	21.02

( $^{125}\text{I}$ -12-Tyr-gFPA as Tracer)

<u>CONTROL</u>	GP22	GP23	<u>INFECTED</u>	GP24A	GP26
D0	6.54 ng/ml	13.26 ng/ml	D0	11.21 ng/ml	6.86 ng/ml
D2	5.21	5.15	D2	4.52	43.66
D5	18.00	4.83	D5	6.62	32.38
D7	51.01	8.74	D7	5.14	325.66
D9	6.65	75.01	D7	Died	8.96

Cross-reactivity of fragments D and E: The elution profile of the 6-hr plasmin digest of guinea pig fibrinogen through a DEAE (DE52) column showed two main peaks in the eluates (Fig. 7). The pooled fraction under each peak contained primarily D and E fragments, respectively. The fraction containing E fragment was dialyzed against 0.5 M monobasic sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ) and further purified by elution through DE52 ion-exchange cellulose with 0.5 M  $\text{NaH}_2\text{PO}_4$ . Isolated D and E fragments were pure by the criteria of immunoelectrophoresis and sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Using  $^{125}\text{I}$ -5-Tyr-gFPA as a radioactive tracer, R125 antiserum showed the greatest sensitivity toward gFPA. The working range for gFPA was between 0.76 to 3.0 pmole/ml (Fig. 8). Guinea pig fibrinogen, D and E fragments cross-reacted extensively with  $^{125}\text{I}$ -5-Tyr-gFPA against R125 anti-gFPA-BSA antibody. Fragment D inhibited the antigen-antibody reaction between 5-Tyr-gFPA tracer and R125 antibody from 30 pmole/ml to 7.5 nmole/ml; whereas fragment E cross-reacted to a slightly lesser extent in the RIA system, from 30 pmole/ml to 30 nmole/ml.

### DISCUSSION

Pichinde virus, a Colombian arenavirus that does not cause disease in humans, has been adapted to inbred guinea pigs in an effort to provide a realistic model for AVHFs, such as Lassa fever (4). A variety of significant hemostatic impairments were found in the experimental infection of strain 13 guinea pigs with Pichinde virus (5). Serum FDP levels progressively increased in infected guinea pigs and reached levels  $>100\text{ }\mu\text{g/ml}$  by day 10 post-infection. Presumably, the increase in serum FDP levels was due to enhanced intravascular coagulation and fibrinolysis in infected animals, suggesting of DIC. Hence, a rise in plasma gFPA levels was anticipated as the Pichinde virus infection progressed in inbred animals. However, an increase in the plasma levels of gFPA liberated from fibrinogen was not detected uniformly in Pichinde-infected animals by the gFPA-RIA using  $^{125}\text{I}$ -5-Tyr-gFPA as a tracer (Tables II and III). On the other hand, an increase in the plasma gFPA could be detected in the majority of the infected animals by the gFPA-RIA using  $^{125}\text{I}$ -12-Tyr-gFPA. All the animals except one (GP24A in Table II) showed an increase in gFPA levels (measured by  $^{125}\text{I}$ -12-Tyr-gFPA tracer) as the virus infection progressed.

In the validation experiments to demonstrate the release of gFPA after the thrombin treatment of guinea pig fibrinogen, gFPA was found to be unstable at 37°C despite the stabilizing effect of EDTA. However,  $^{125}\text{I}$ -12-Tyr-gFPA appeared to be less susceptible to proteolytic cleavage (by thrombin?) than  $^{125}\text{I}$ -5-Tyr-gFPA since, in yield, roughly 73 times gFPA was detected using the former tracer, after the 20-min thrombin cleavage of guinea pig fibrinogen (see Figs. 5 and 6). The results of the in vitro thrombin release of gFPA from fibrinogen could conceivably explain the gFPA data derived from Pichinde-infected animals. In short, the thrombin enzymes of human and guinea pig are both capable of degrading the two tracers used in the gFPA-RIA. Apparently,  $^{125}\text{I}$ -12-Tyr-gFPA is affected less by the thrombin degradation than the other tracer in the gFPA-RIA.

In general, each anti-gFPA-BSA serum was consistent in the pattern of immunoreactivity. R125 antiserum, which exhibited the highest sensitivity toward gFPA and was used extensively in these studies, was produced in a rabbit immunized with gFPA-BSA conjugate via the glutaraldehyde method. In the gFPA-RIA system employing  $^{125}\text{I}$ -12-Tyr-gFPA, the sensitivity of R125 antiserum was relatively less compared with the assay system utilizing  $^{125}\text{I}$ -5-Tyr-gFPA (Figs. 1 and 2). This result suggests that the epitope of R125 anti-gFPA antibody may reside in the C-terminal half of the 13-amino acid residue-peptide. Guinea pig FPA lacks lysine; consequently, glutaraldehyde would couple gFPA via the  $\text{NH}_2$  group of N-terminal threonine to the  $\text{NH}_2$  groups of lysine residues in BSA. The resultant conformation of gFPA-BSA conjugate would be such that the C-terminal half of gFPA is likely to be an immuno-dominant group.

The specificity of R125 antiserum can explain the difference between the RIA data obtained with the assay system utilizing  $^{125}\text{I}$ -5-Tyr-gFPA and  $^{125}\text{I}$ -12-

Tyr-gFPA. Presumably, guinea pig thrombin is capable of cleaving the tracer in the mid-section beyond  $^{125}\text{I}$ -5-Tyr, most likely at the carboxyl end of glutamic acid-6. As a consequence, in the RIA using  $^{125}\text{I}$ -5-Tyr-gFPA, the C-terminal half of gFPA, which contains the epitope of R125 anti-gFPA antibody, is rapidly cleaved off from the  $^{125}\text{I}$ -5-Tyr residue. However, this thrombin cleavage does not affect the RIA using  $^{125}\text{I}$ -12-Tyr-gFPA.

The RIA data presented in this report lead us to the inescapable conclusion that there is transient elevation of the plasma gFPA level in response to Pichinde virus infection, which we interpret as evidence of in vitro thrombin activity and thrombin generation. Lee et al. (24) reported the presence of transitory DIC in human patients affected with Korean hemorrhagic fever (hemorrhagic fever with renal syndrome). Possibly, Pichinde-infected guinea pigs also experience the coagulopathy of short duration, as indicated by increased thrombin activity.

### CONCLUSIONS

Hemorrhagic viruses can alter hemostasis by activating immune and inflammatory pathways (25). Immune and inflammatory mediators such as interleukin-1 and tumor necrosis factor have physiologic activities that modulate hemostatic functions (26,27), whereas on the other hand, activated coagulation factors can induce inflammation as well as alter the immune response (28,29). Immune effector cells, e.g. monocytes, are simultaneously hemostatic effector cells, making it impossible to dissect one activity from the other (30).

The pathophysiologic mechanism by which significant coagulation abnormalities are induced in the experimental infection of strain 13 guinea pigs with Pichinde virus has not been completely elucidated. Experimental evidence presented in this report indicates that Pichinde-infected guinea pigs experience the coagulopathy of short duration, similar to transitory DIC in human patients affected with Korean hemorrhagic fever (24). Understanding of the complex interplay among the three physiologic responses to arenaviral infection, i.e., hemostasis, immunity and inflammation, may shed light on the pathogenesis of Pichinde virus infection.

As Cosgriff stated in his succinct review on virally-induced DIC (25), "in attempting to analyze the relative importance of DIC and other mechanisms of hemorrhage in the viral hemorrhagic fevers, it is important to keep in mind that some of these diseases show considerable variability in hemorrhagic manifestations from one geographic area to another and even from one time period to another. This variability undoubtedly reflects differences in viral strains." In AVHFs, it appears that DIC occurs only infrequently. Peters et al. (31) stated that DIC does not appear to be a central pathogenic mechanism in Lassa fever. Lange et al. (32) utilized the experimental infection of rhesus monkeys with Lassa virus to study hematologic and hemostatic aspects of

Lassa fever. They concluded that DIC does not play any role in this animal model of Lassa fever.

An assortment of physiologic events are observed in Pichinde-infected animals which are of major clinical importance (31, Table IV).

TABLE IV. Physiologic Changes in Pichinde-Infected Strain 13 Guinea Pigs

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Weight loss/wasting (>25%)  
Cardiac decompensation  
Acidosis (pH 7.29, pCO<sub>2</sub> 28 mmHg, no anion gap)  
Renal tubular defect (alkaline urine, decreased TmPAH, decreased Tm glucose, increased C PAH)  
Adult respiratory distress syndrome/shock lung  
Circulatory response to fluid load: crystalloid worsens, colloid temporarily improves

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Tm = Tubular maximal excretory capacity  
PAH = p-Aminohippurate  
C = Clearance

The assorted physiologic changes that occur during the Pichinde-infection of inbred guinea pigs do not point to DIC as the major pathogenic mechanism of this disease. However, the hemostatic derangement can trigger the changes in immune and inflammatory pathways. Clearly, additional studies are needed on the interaction between the coagulation pathway and physiologic mediators such as interleukins, platelet activating factor, cachectins and other soluble mediators.

Luterman et al. (33) intravenously infused one of the fibrinogen degradation products, i.e., fragment D, into rabbits. Four hours after infusion, the animals displayed a clinical pathological pattern which closely resembled acute respiratory distress syndrome (ARDS), including hypoxia, thrombocytopenia, increased pulmonary capillary permeability, interstitial edema, hypertrophy of alveolar lining cells, and intra-alveolar hemorrhage. The ARDS



is one of the major clinical symptoms exhibited by Pichinde-infected inbred guinea pigs. We have previously observed high serum levels of FDP in the experimental infection of strain 13 guinea pigs with Pichinde virus (5). Belew et al. (34) have isolated some plasminic degradation products of fibrin, i.e., a pentapeptide and an undecapeptide, which increased vascular permeability. Also, some elastase degradation products from fibrin have been isolated, which have a potent permeability increasing effect (35). Since elastase is found in large amounts in the alveoli of patients with ARDS, it is quite possible that these plasmin or elastase degradation products of fibrin could induce a permeability increase in guinea pigs, leading to ARDS. Studies in these directions are presently underway in this laboratory.

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APPENDIX

1. Articles Published:

The work related to the following articles received the indirect support from U.S. Army Grant DAMD 17-89-Z-9005. These papers acknowledge the partial support of the above grant.

Chen, JP, Robinson R, Lu TS, Enderson BL. Fibrin(ogen) degradation products in the coagulopathies of trauma patients. In: Matsuda M et al., eds. Fibrinogen 4 - Current basic and clinical aspects. Amsterdam: Elsevier Science Publishers, 1990:195-199. (Enclosed)

Enderson BL, Chen JP, Robinson R, Maul KI. Fibrinolysis in multisystem trauma patients. J Trauma 1991;31:1240-1246. (Enclosed)

2. Abstracts Published:

Chen JP, Guinn LC, Schroeder EC. Radioimmunoassay of guinea pig fibrinopeptide A and cross-reactivity with fibrinogen and fibrin(ogen) degradation products. Fibrinolysis 1990;4, Suppl 3:32. (enclosed)

Chen JP, Marsh LC, Schroeder EC. Radioimmunoassay of guinea pig fibrinopeptide A: A means to deduce the pathogenesis of Pichinde virus infection. FASEB J 1991;5:A524. (enclosed)

3. Articles to be Submitted:

Chen JP, Marsh LC. Radioimmunoassay of guinea pig fibrinopeptide A and cross-reactivity with fibrin(ogen) degradation products. Biochimica et Biophysica Acta (1992).

Chen JP, Schroeder EC, Enderson BL, Maul KI. Pichinde virus infection of strain 13 guinea pigs: Radioimmunoassay of fibrinopeptide A to assess thrombin action in infected animals. Journal of Infectious Diseases (1992).

4. Personnel Supported by the Grant:

Marsh, Lois C; Senior Research Technician, 02/13/89-06/17/91.

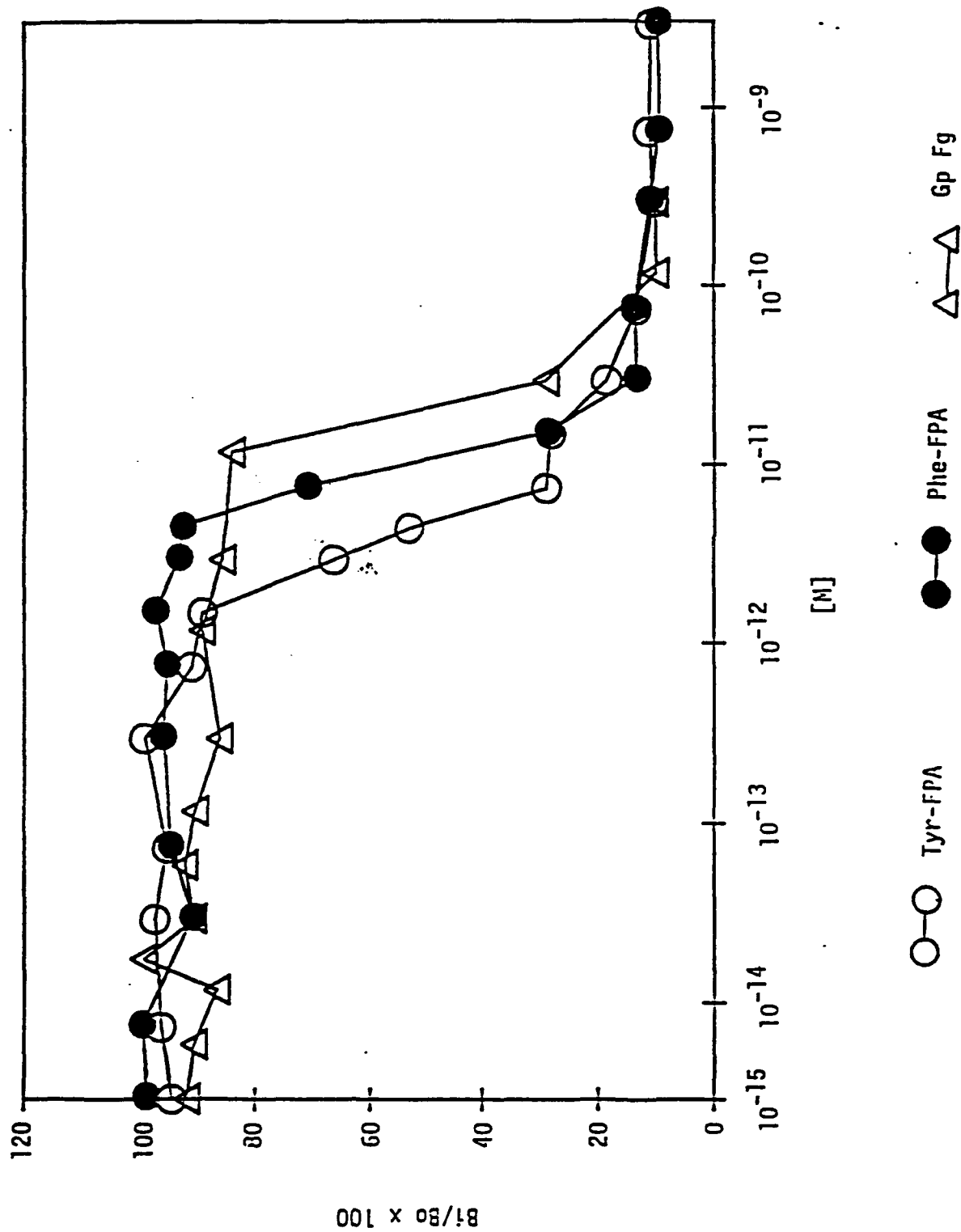


Fig. 1: Competitive inhibition profile of gFPA, 5-Tyr-gFPA and GP fibrinogen in the assay system involving <sup>125</sup>I-5-Tyr-gFPA and R125 anti-gFPA-BSA.

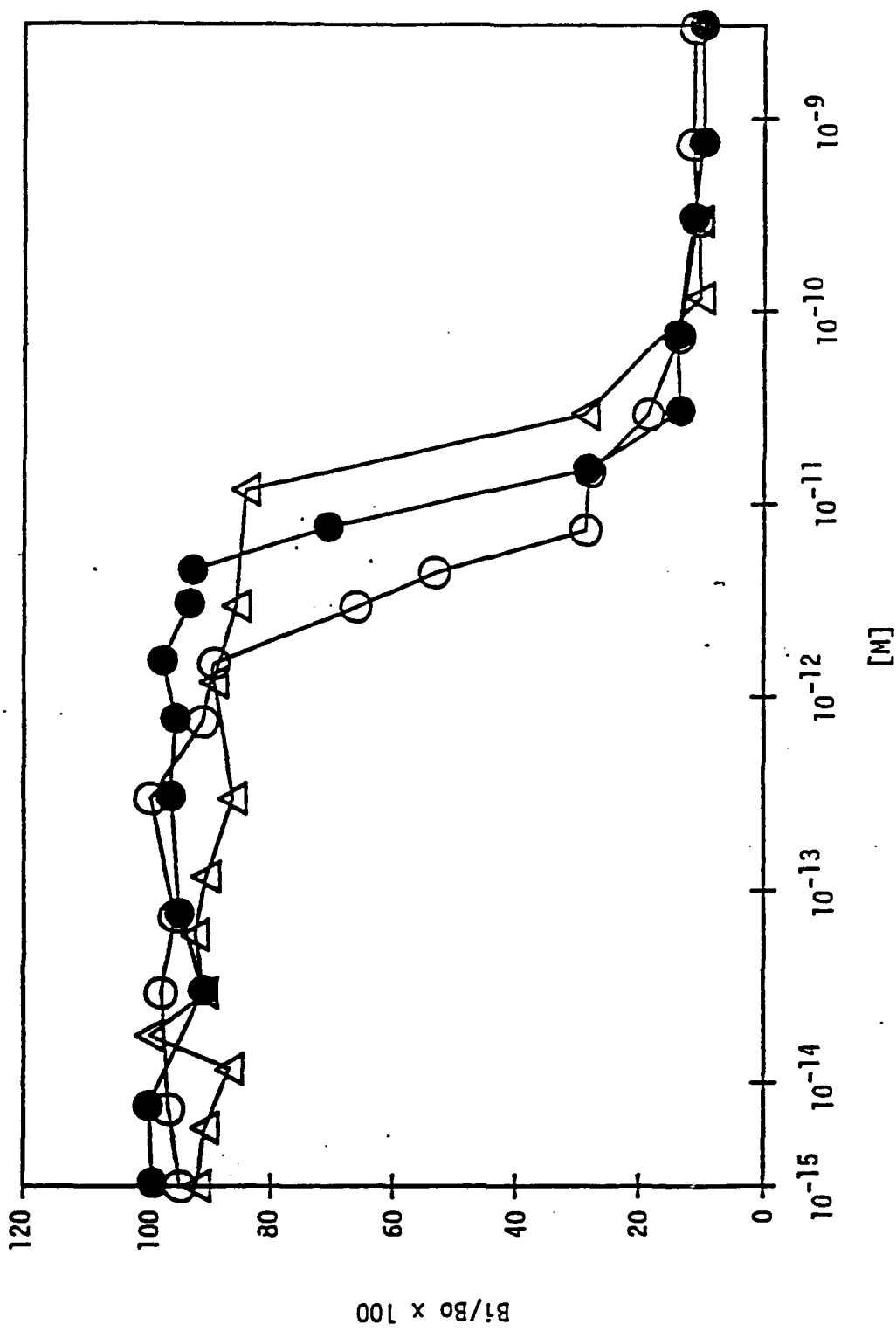


Figure: 2

Fig. 2: Competitive inhibition profile of gFPA, 12-Tyr-gFPA and GP fibrinogen in the RIA involving <sup>125</sup>I-12-Tyr-gFPA and R125 anti-gFPA-BSA serum.



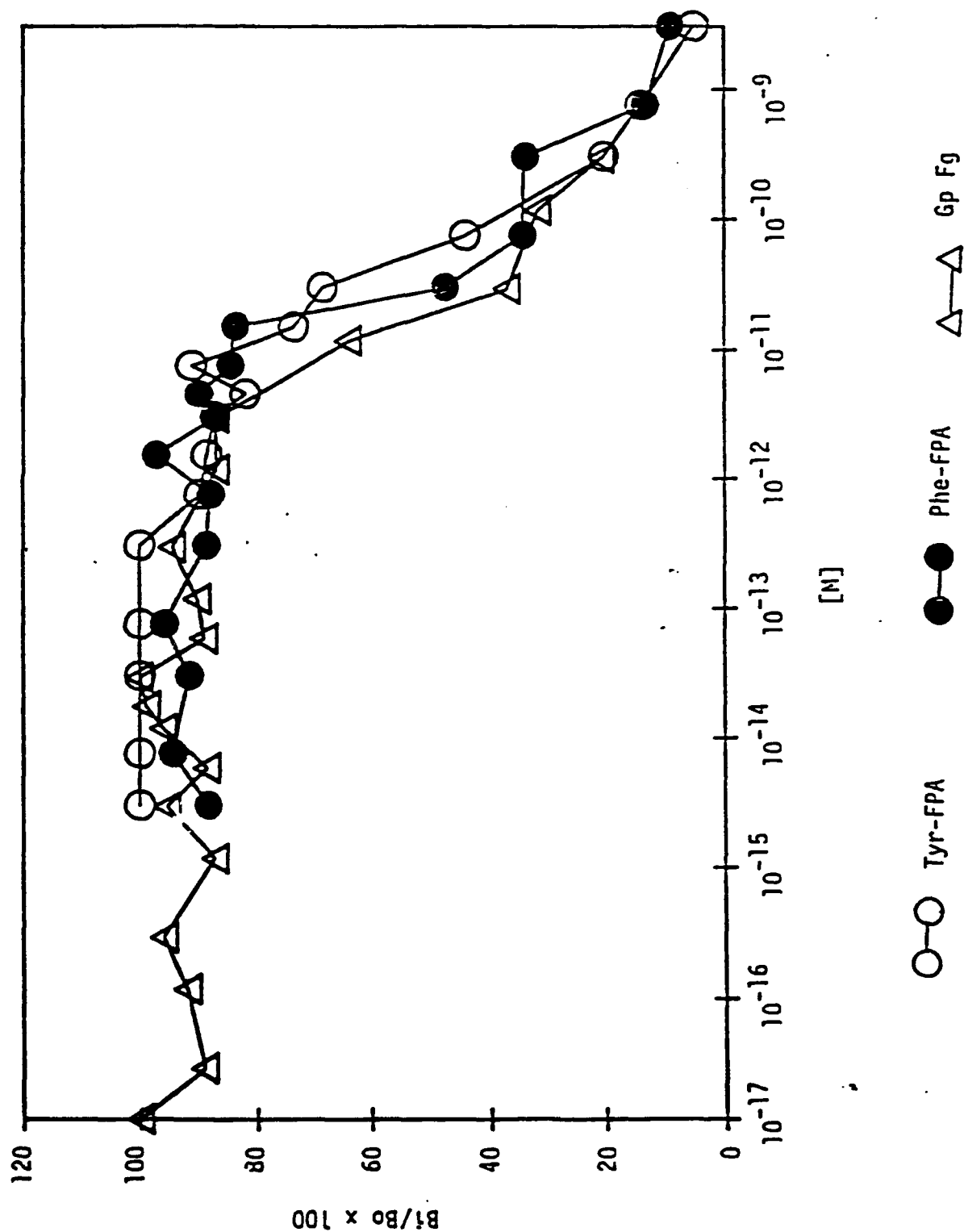


Fig. 3: Competitive inhibition profile of gFPA, 5-Tyr-gFPA and GP fibrinogen in the assay system involving  $^{125}\text{I}$ -5-Tyr-gFPA and R130 anti-gFPA-BSA serum.

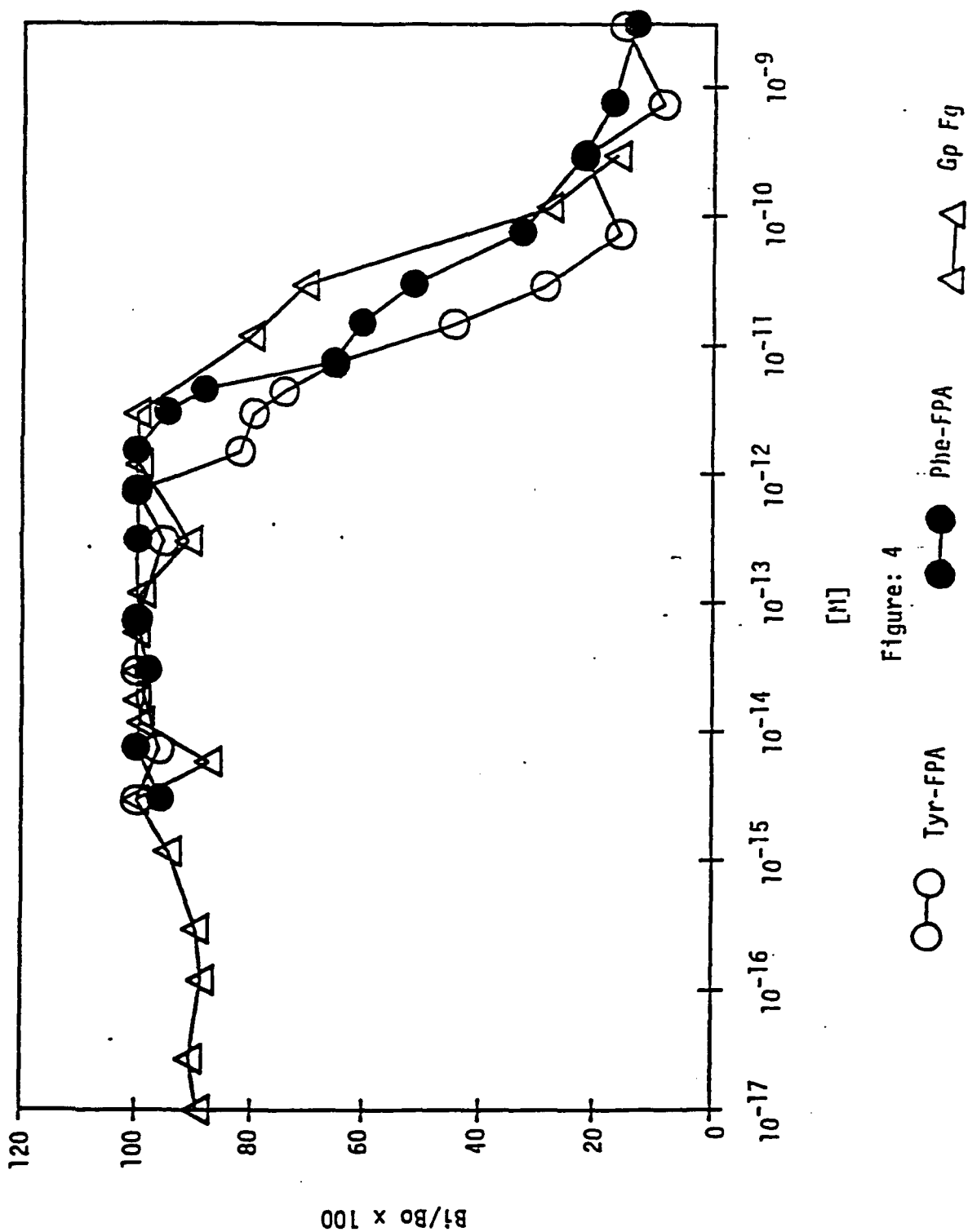


Figure: 4

Fig. 4: Competitive inhibition profile of gFPA, 12-Tyr-gFPA and GP fibrinogen in the RIA involving  $^{125}\text{I}$ -12-Tyr-gFPA and R130-gFPA-BSA serum.

## Cleavage of gFPA from Fibrinogen by Thrombin

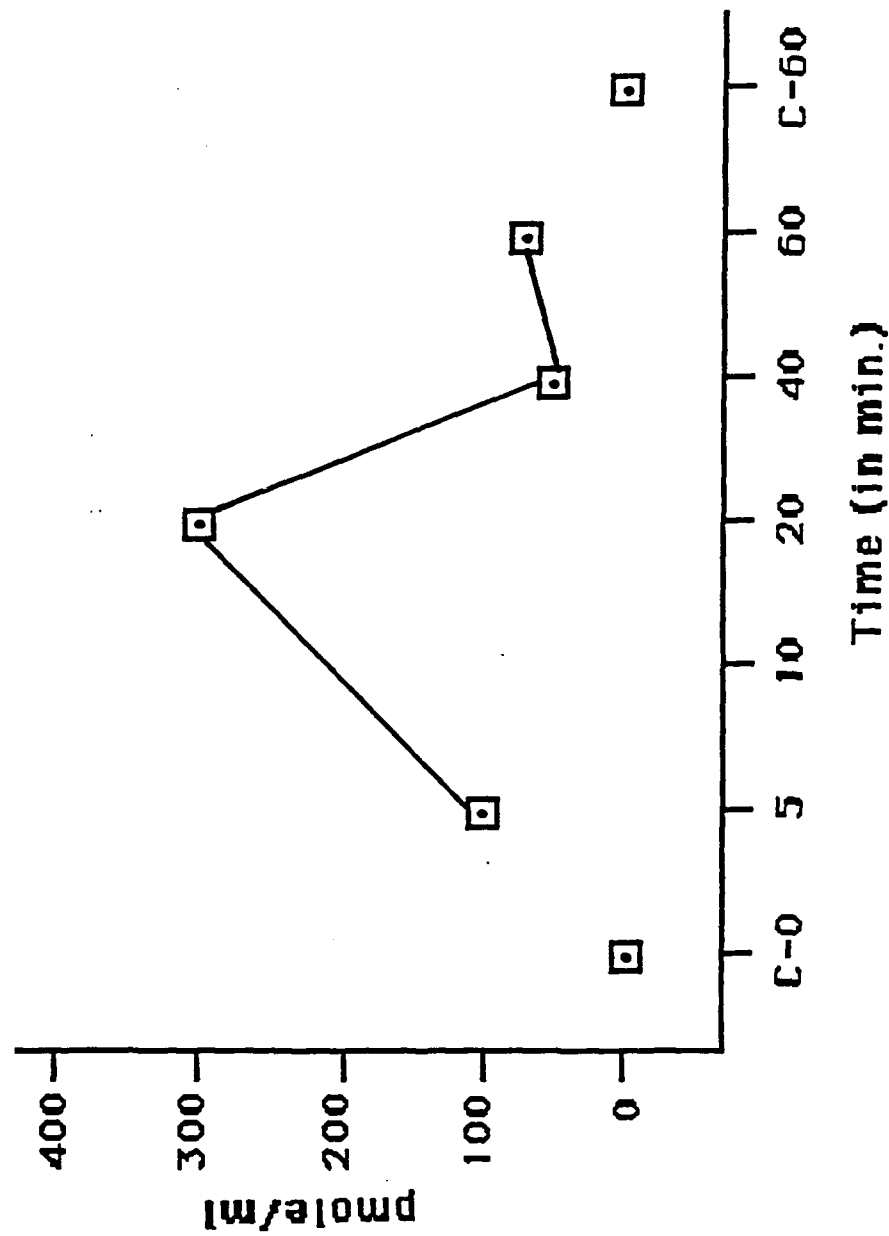


Fig. 5: Time course for the cleavage of gFPA from guinea pig fibrinogen by human thrombin. The assay system is based on the inhibition of gFPA in the reaction between  $^{125}\text{I}$ -labeled 5-Tyr-gFPA and R125 anti-gFPA-BSA antibody.

## Cleavage of gFPA from Fibrinogen by Thrombin

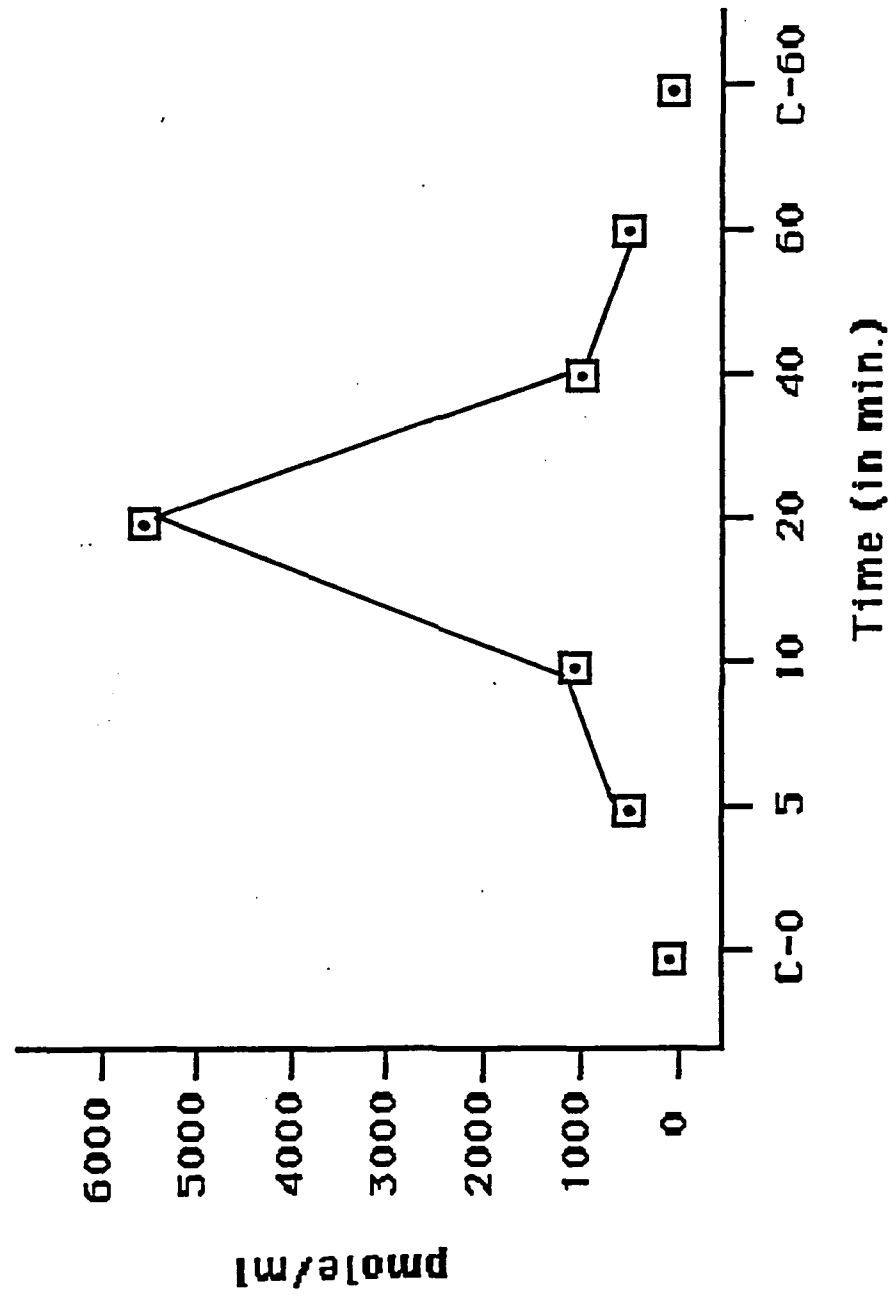


Fig. 6: Time course for the cleavage of gFPA from guinea pig fibrinogen by human thrombin. The gFPA was assayed in the gFPA-RIA involving  $^{125}\text{I}$ -labeled 12-Tyr-gFPA and R125 anti-gFPA-BSA antibody.

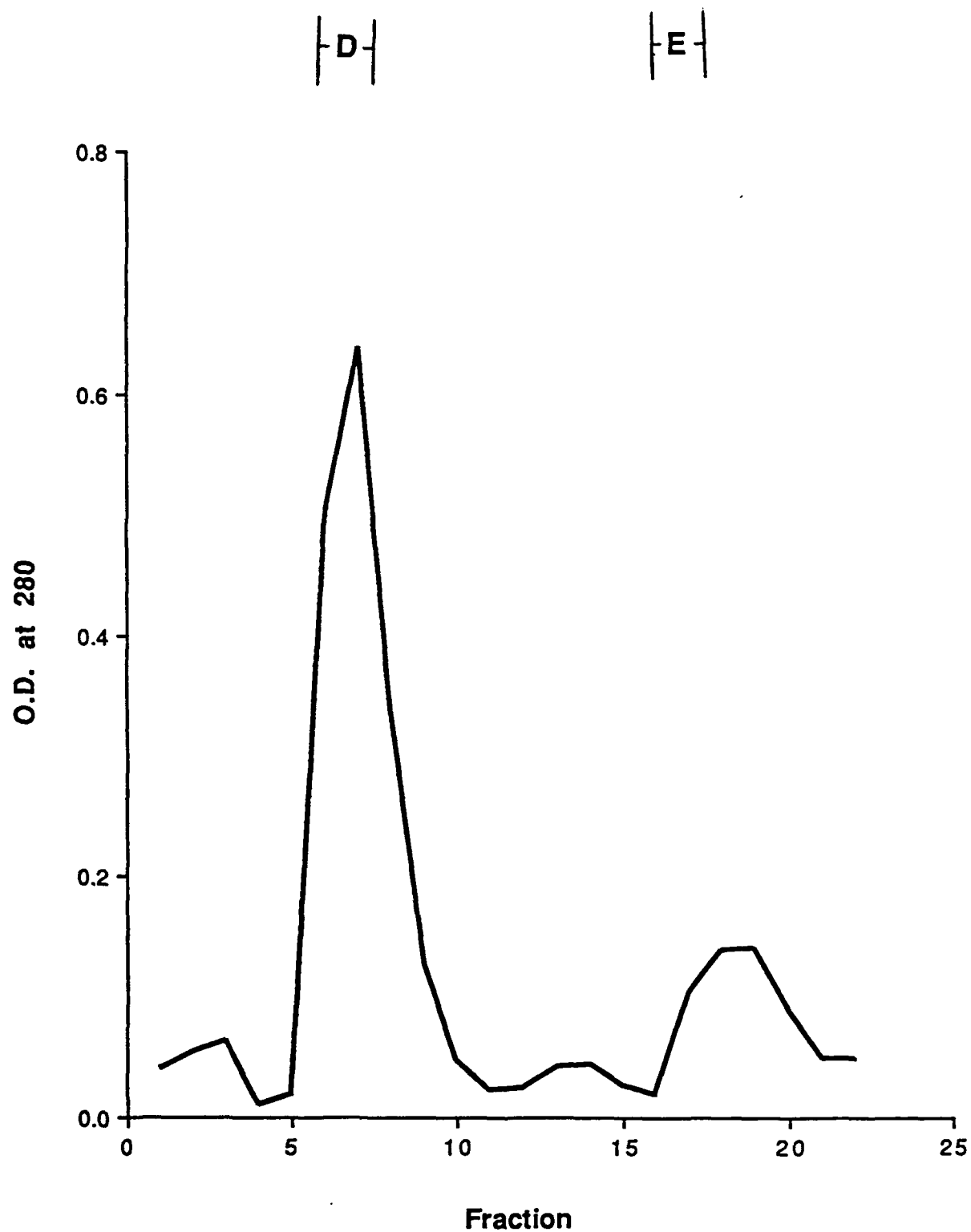


Fig. 7: Elution pattern obtained after chromatography of guinea pig FDP on DEAE-cellulose. A linear concentration gradient of phosphate buffer was used to elute the proteins.

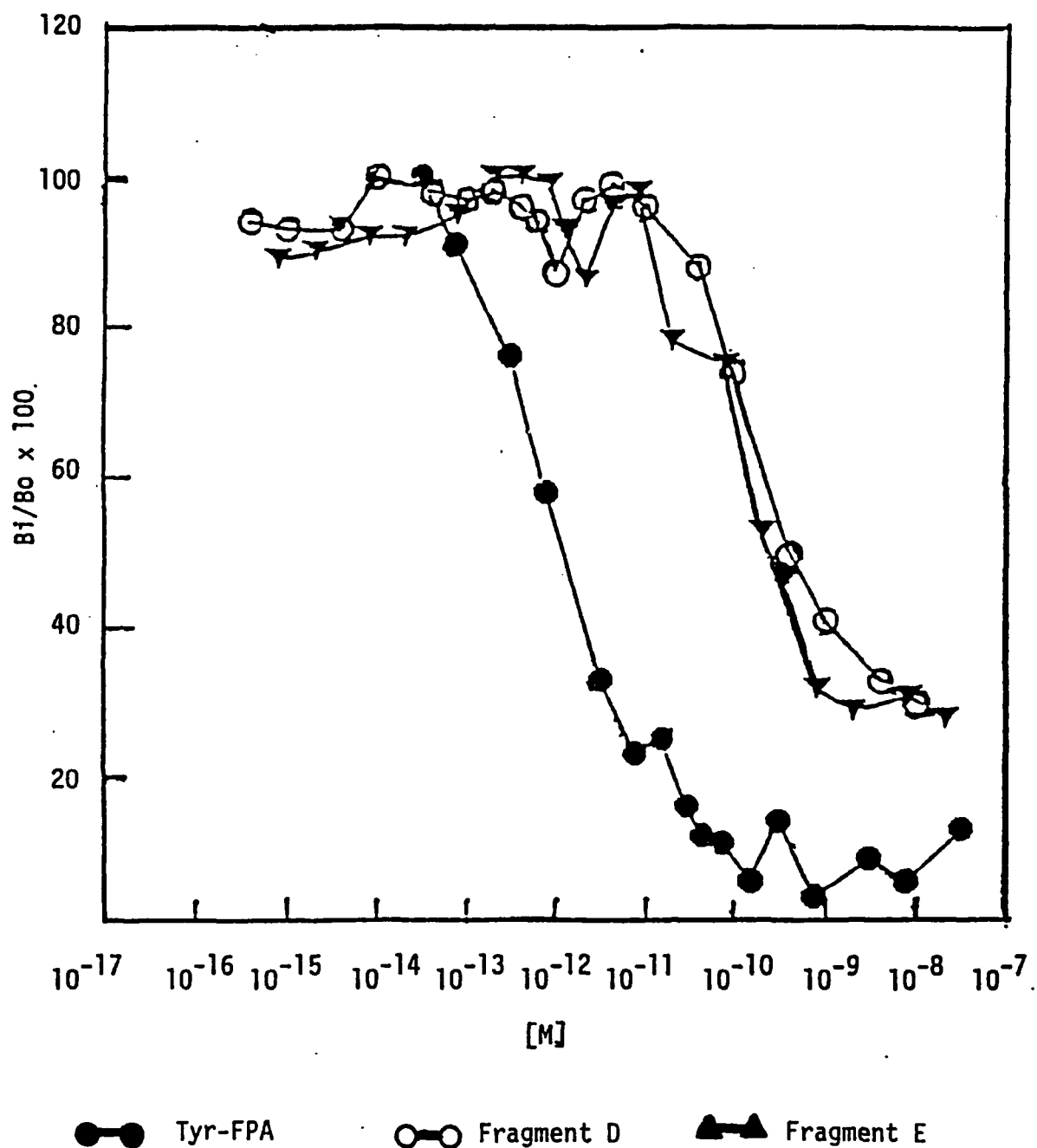


Fig. 8: Competitive inhibition assay for the expression of gFPA epitope present on the synthetic 5-Tyr-gFPA, fragments D and E. The assay system consisted of <sup>125</sup>I-5-Tyr-gFPA and R125 anti-serum.

## FIBRIN(OGEN) DEGRADATION PRODUCTS IN THE COAGULOPATHIES OF TRAUMA PATIENTS

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### INTRODUCTION

Trauma patients often have coagulation disorders that predispose them to bleeding and thrombosis. Laboratory indices of thrombin and plasmin formation may be related to the severity of acute injuries (1,2). We studied 42 consecutive adult patients (23 males and 19 females) on arrival, and 24 and 48 hours following their admission to the intensive care unit (ICU). The following coagulation and fibrinolytic parameters were measured: (a) prothrombin time (PT); (b) activated partial thromboplastin time (APTT); (c) antithrombin III (AT-III); (d) plasminogen activators by fibrin plate assay and (e) D-dimer crosslinked fibrin fragments by latex agglutination test (LAT) and enzyme linked-immunosorbent assay (ELISA).

### MATERIALS AND METHODS

A blood sample was withdrawn initially at the emergency room from femoral vein and subsequently from an antecubital vein at ICU. Nine ml of venous blood was mixed with 1 ml of 0.1 M sodium citrate in a siliconized glass tube. The blood was centrifuged at 2600 rpm for 20 min and the plasma centrifuged at 10,000 rpm for 20 min at 4°C. Plasma samples were aliquoted and "snap-frozen" in ethanol-dry ice before being stored at -50°C.

The presence of crosslinked fibrin degradation products (XDP) was detected using the LAT kit supplied by American Dade, Miami, FL (3) and the ELISA kit purchased from American Diagnostica, New York, NY (4). The D-dimer LAT provides for an agglutination test using latex particles coated with a monoclonal antibody to D-dimer (DD-3B6). The D-dimer ELISA uses the same monoclonal antibody as the D-dimer LAT for a capture antibody and a second, less specific, monoclonal antibody for enzyme labeling. PT and APTT were measured with commercial reagents.

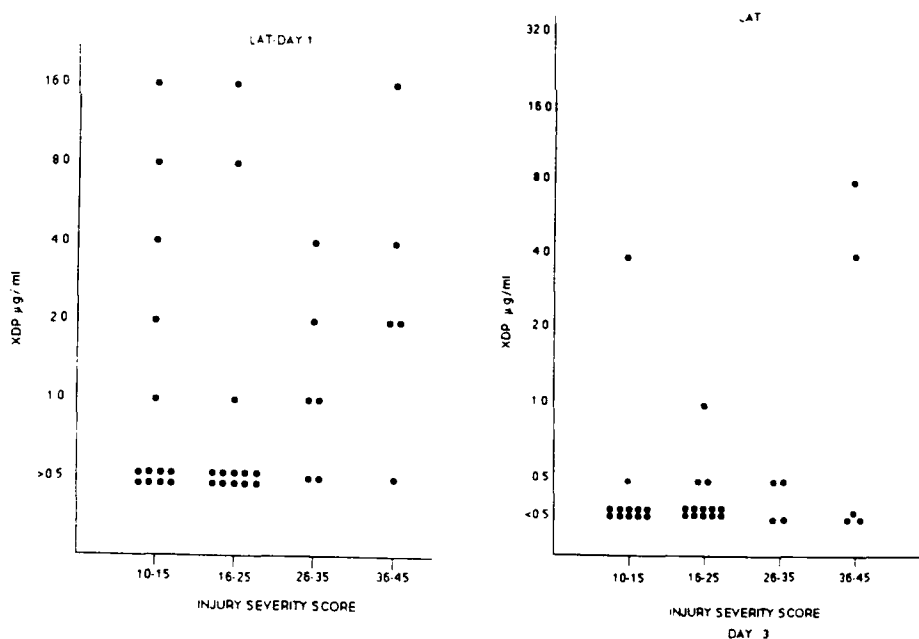
Plasma AT-III levels were determined by chromogenic substrate (S-2238) assay employing the Coatest Antithrombin kit supplied by Kabi Vitrum Diagnostica. Plasminogen activator (PA) activity was determined by a modification of the fibrin plate assay of Kluft (5). For preparation of fibrin plates, 0.2% (w/v) human fibrinogen (Kabi, Stockholm, Sweden) and 0.53% (w/v) agarose (indubiose A37, Accurate Chemical & Scientific Corp., Westbury, NY) in barbital buffer (pH 7.3, ionic strength 0.15) were used. Fibrinogen and agarose solutions were

mixed in 1:1 ratio and 15 ml of this mixture was poured into petri dishes (9 cm diameter). Fibrinogen was clotted with bovine thrombin in a final concentration of 0.7 NIH units/ml. After solidification, 6 wells with the diameter of 5 mm were cut into the plate. Euglobulin was precipitated from 0.2 ml of plasma added to a chilled mixture composed of 4.5 ml water, 0.05 ml 1% acetic acid, and 0.2 ml dextran sulfate solution (100 mg/L). Euglobulin fraction was redissolved in 0.2 ml of ethylenediamine tetraacetate (EDTA) buffer, pH 7.8, prepared as described previously (5). Twenty-five  $\mu$ l of euglobulins was placed into wells in triplicate and incubated for 18 hours at 37°C.

## RESULTS

### D-Dimer equivalent of XDP

In blood samples drawn from 12 normal donors (6 males and 6 females), D-dimer equivalents of XDP, determined by D-dimer ELISA, ranged from 10 to 54 ng/ml and had a mean D-dimer level  $\pm$  SD of  $22.9 \pm 14.8$  ng/ml. The plasma D-dimer values measured by LAT indicated the immunoreactives of less than 0.5  $\mu$ g/ml for 12 normal individuals.



Figs. 1 and 2. Plasma levels of XL-fibrin degradation products (XDP) on Days 1 and 3 of trauma patients. The patients are grouped together by injury severity score (ISS).

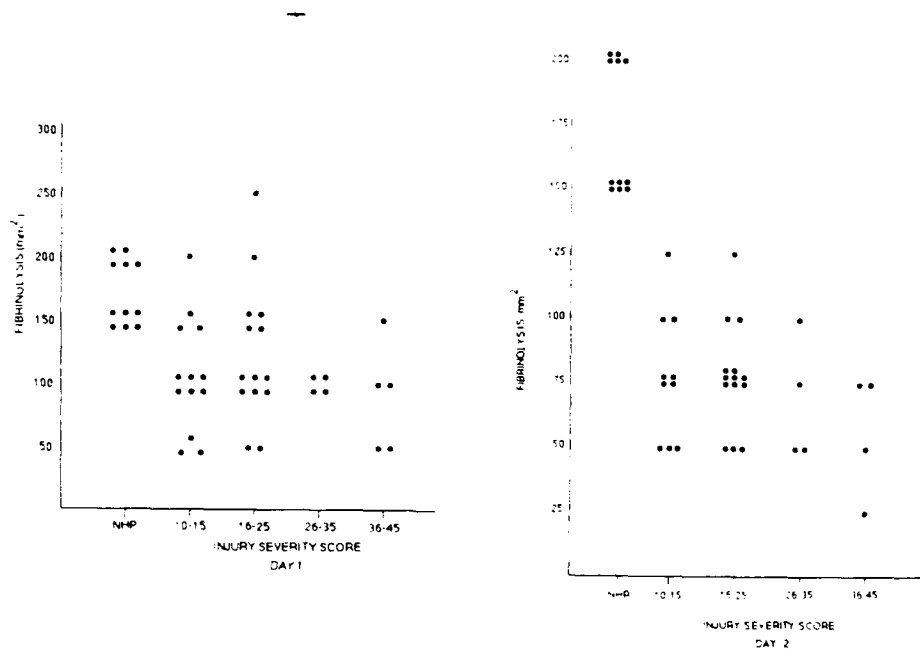


Injury severity score (ISS) for each trauma patient is based on the abbreviated injury score (AIS) assigned to six body regions (i.e., head/neck, face, chest, abdomen, extremities, and skin).  $ISS = (AIS-1)^2 + (AIS-2)^2 + (AIS-3)^2$ . ISS is calculated from AIS assigned to three most severely injured body regions. Changes in the D-dimer equivalent of XDP in relation to ISS, as measured by D-dimer LAT, are illustrated in Figures 1 and 2. In all ranges of injury severity, a proportion of patients showed increased D-dimer values in the first day after trauma. In the third day, these elevated D-dimer immunoreactivities were considerably reduced to the normal values.

The D-dimer equivalent of XDP was also measured by D-dimer ELISA using monoclonal antibody DD-3B6 in the plasma samples of all trauma patients. The D-dimer values, as measured by LAT and ELISA, showed a highly significant correlation by Spearman rank correlation (0.69,  $p = 0.0001$ ). However, ISS and D-dimer immunoreactivities, determined by D-dimer ELISA, did not reveal any significant relationship.

#### PA activity by fibrin plate

Changes in the PA activity in relation to severity of injury (ISS), as measured by the fibrin plate method, are depicted in Figures 3 and 4. Of the 42



Figs. 3 and 4. PA activity on Days 1 and 2 determined by the fibrin plate assay, expressed as diameters square. NHP: Normal human plasma. The patients are grouped together by ISS.

trauma patients, 4 patients exhibited higher than normal PA activity at the time of admission to the ICU. The remainder had suppressed PA activity, compared to normal subjects on Day 1. Of the 32 patients, who had fibrinolysis data on all three days, 4 had the lowest PA activity on Day 1, 14 had the lowest PA on Day 2, and 14 had the lowest PA on Day 3. This suppression of PA activity correlated significantly by Spearman rank correlation with D-dimer equivalent of XDP determined by ELISA on the same day ( $-0.34$ ,  $p = 0.03$ ). Furthermore, the correlation of PA suppression was even more significant ( $-0.45$ ,  $p = 0.0055$ ) with maximum D-dimer levels of Days 1 to 3. On the average, PA activity of all trauma patients showed the greatest suppression on the second day, but daily PA activities of most patients were below normal.

With the exception of 4 patients in PT and 8 patients in APTT, both PT and APTT did not deviate appreciably from normal controls. These deviations were rather random in nature and did not correlate well with severity of injury (ISS) or D-dimer levels. AT-III levels did not deviate markedly from normal controls.

It appears that the elevation of D-dimer levels was strongly associated with head and spinal cord injuries. Two patients with less severe injuries but evidence of increased suppression of PA activity and elevation of D-dimer levels developed clinical thrombosis, including a fatal pulmonary embolism in one.

#### DISCUSSION

Previous studies have shown that trauma can produce changes in the blood compatible with a hypercoagulable state in the first several hours after injury (1,6). The Day 1 data on XDP resulting from the plasmin action on crosslinked fibrin indicate that fibrinolytic activity in the blood increases in response to a hypercoagulable state induced by injury. Our data on plasma D-dimer levels are also compatible with the data on plasma X-oligomers in 13 trauma patients reported by Garcia Frade *et al.* (7). However, the actual numerical values of D-dimer immunoreactivities showed a considerable variation between individuals and, contrary to expectation, showed no correlation with the degree of injury (ISS).

Recently, Brenner *et al.* (8) found that the concentration of soluble cross-linked fibrin polymer is directly related to the D-dimer immunoreactivity determined by monoclonal antibody DD-3B6 after plasmin digestion. It is possible that the elevation of D-dimer levels observed in trauma patients may be related to the plasmic degradation of soluble crosslinked fibrin rather than the actual fibrinolysis of thrombus.

A decrease in PA activity was found in the majority of trauma patients and was not significantly correlated with severity of injuries. Suppression of

fibrinolysis was correlated with the elevation of D-dimer levels and reached its maximum 24 and 48 hours after admission to ICU. A limiting factor in this study might be the elapsed time between injury and admission to hospital. In the majority of patients, an increase in PA activity was not observed, while Bain (6) stated that an increase in PA activity occurred several hours after injury.

Interestingly, the elevation of D-dimer immunoreactivity appears to be more closely related to types of injury and subsequent clinical complications (e.g., hemorrhage and thrombosis). It has been estimated that the patient with an acute head or spinal cord injury has a reported 40% incidence of deep vein thrombosis and a greater than 1% incidence of pulmonary embolism (9). One patient with less severe injuries but with a previous history of hypertension gave evidence of increased consumption of fibrinolytic components and developed a fatal pulmonary embolism in the course of treatment. Thus, D-dimer LAT and fibrin plate assay, which are relatively easily obtainable measurements of fibrinolytic activity in blood, may be more clinically useful.

#### ACKNOWLEDGEMENTS

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## Fibrinolysis in Multisystem Trauma Patients

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KIMBALL I. MAULL, MD

Changes in the fibrinolytic system may lead to coagulation disorders in acute trauma patients. This study examined fibrin degradation by measuring D-dimer crosslinked fibrin degradation products (indicates hypercoagulability), plasminogen activators (fibrinolysis), and antithrombin III in 42 adult trauma patients and correlated these data with injury severity, types of injury, complications, and clinical tests of coagulation. Hypercoagulability and suppression of fibrinolysis were seen in most patients and were not correlated with severity of injury. These changes appeared most severe in patients with nervous system injury. Several patients with less severe injuries but evidence of hypercoagulability developed clinical evidence of pathologic thrombosis. Latex agglutination of D-dimer provides a rapid test of fibrinolysis that may be clinically useful in the management of trauma patients who cannot be easily studied for thrombosis.

Normal hemostasis involves a complex balance between the formation of fibrin (coagulation) and the degradation of fibrin (fibrinolysis).<sup>1</sup> This system is necessary to control hemorrhage from injured vessels and promote later recanalization. Imbalance in the production and degradation of fibrin can lead to complications of excessive bleeding or thrombosis. The trauma patient is susceptible to these disorders because of significant tissue injury, which initiates coagulation, mobilizes acute phase proteins to compensate for the injury, and depletes factors necessary for coagulation/fibrinolysis.<sup>2</sup>

Studies to correlate changes in the coagulation system with the severity of trauma sustained have had variable results because of the lack of specificity of the tests, the effects of resuscitation, blood product transfusion, and the different hemostatic mechanisms available for coagulation.<sup>3</sup> Similarly, studies of the fibrinolytic system in trauma have produced conflicting results because of the lack of specificity of tests of fibrinolysis, the timing of the measurements, and the many activators and inhibitors involved.<sup>2,4,5</sup> Measurement of fibrin degradation products (FDP) can be misleading, since this test detects breakdown products of fibrinogen, fibrin monomer, and fibrin polymers (both soluble and insoluble). More specific information about fibrin breakdown can be obtained

by measuring D-dimer crosslinked fibrin degradation products (XDP), which result only from the degradation of insoluble crosslinked fibrin clots. The standard measure of XDP has been an ELISA technique, but this is time-consuming and therefore not clinically useful. A recently developed assay for XDP offers the advantage of specificity for breakdown of fibrin as well as rapid turnaround time.<sup>6</sup>

The present study was designed to prospectively evaluate coagulation and fibrinolysis in patients who sustained multiple system trauma severe enough to require a stay in an intensive care unit (ICU). Changes in the fibrinolytic system were correlated with injury severity, causes of trauma, types of injuries, complications, and standard coagulation studies. Two different means of measuring D-dimer XDP were utilized to determine their applicability.

### METHODS

Forty-two consecutive injured adult patients (23 men and 19 women) requiring ICU care were admitted to this study over a 3-month period. A blood sample was drawn with the initial trauma laboratory samples in the emergency unit, usually via femoral puncture. Subsequent samples were drawn in the intensive care unit from peripheral veins or arterial lines at 24 and 48 hours after injury. The design of the study was approved by the Institutional Review Board and informed consent was obtained.

Nine milliliters of blood was mixed with 1 mL of 0.1 mol/L sodium citrate in a siliconized glass tube. The blood was centrifuged at 2,600 rpm for 20 minutes and the plasma centrifuged at 10,000 rpm for 20 minutes at 4°C. Plasma samples were aliquoted and "snap-frozen" in ethanol-dry ice before being stored at -50°C. Several variables of coagulation and fibrinolysis were determined from the samples, including antithrombin III (AT-III), plasminogen activators (PA) by fibrin plate assay,

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and D-dimer XDP by both a latex agglutination test (LAT) and an enzyme-linked immunosorbent assay (ELISA). These data were compared with clinical tests of coagulation, including prothrombin time (PT) and activated partial thromboplastin time (APTT), which were obtained routinely on admission, as well as other tests performed as indicated by the patient's clinical course. The data were correlated with severity indices, including the Injury Severity Score (ISS).

Crosslinked fibrin degradation products (XDP) were detected using two methods. The first method uses a LAT kit supplied by American Dade of Miami, Florida.<sup>6</sup> This is an agglutination test that uses latex particles coated with a mouse monoclonal antibody to D-dimer (DD-3B6). The second method uses an ELISA kit from American Diagnostica of New York, NY,<sup>7</sup> which contains the same monoclonal antibody as the LAT kit to serve as a capture antibody and a second, less specific, monoclonal antibody that reacts against epitopes present on both D-dimer and fibrinogen degradation products (fragment D) for enzyme labeling. The advantage of the LAT is its rapidity (30 minutes vs. 4 hours). Data from the two techniques of measuring D-dimer XDP were compared.

Plasma AT-III levels were determined by a chromogenic substrate (S-2238) assay with the Coatest Antithrombin kit supplied by Kabi Vitrum Diagnostica. Plasminogen activator (PA) activity was determined by a modification of the fibrin plate assay of Kluft.<sup>8</sup> Fibrin plates were prepared by combining solutions of 0.2% (wt/vol) human fibrinogen (Kabi, Stockholm, Sweden) and 0.53% (wt/vol) agarose (indubiose A37, Accurate Chemical and Scientific Corp., Westbury, NY) in barbital buffer (pH 7.3, ionic strength 0.15) in a 1:1 ratio. Fifteen milliliters of this mixture were poured into 9-cm-diameter Petri plates and clotted with bovine thrombin in a final concentration of 0.7 NIH units/mL. After solidification, 6 wells (5-mm diameter) were cut into the plate. Euglobulin was precipitated from 0.2 mL plasma added to a chilled mixture composed of 4.5 mL water, 0.05 mL 1% acetic acid, and 0.2 mL dextran sulfate solution (100 mg/L). The euglobulin fraction was redissolved in 0.2 mL ethylenediamine tetra-acetate (EDTA) buffer, pH 7.8, and prepared as previously described.<sup>8</sup> Twenty-five microliters of euglobulins were placed into wells in triplicate and incubated for 18 hours at 37°C.

Blood samples were drawn from 12 normal donors (6 men and 6 women) to establish normal values for D-dimer by both ELISA and LAT. Results were correlated using Spearman rank correlation, with significance being assumed at  $p < 0.05$ .

## RESULTS

### Population Demographics

There were 37 victims of blunt trauma (31 from motor vehicle crashes, 4 from falls, and 2 from miscellaneous causes) and 5 penetrating trauma victims (4 gunshot wounds, 1 stab). Initial Revised Trauma Scores ranged from 6 to 12, with all but seven being 10 or greater. Injury Severity Score (ISS) ranged from 8 to 43, with all but 3 patients having an ISS of 12 or more. Forty of the patients survived. One patient died of a gunshot wound to the head and one died of a massive pulmonary embolism.

Since most of the patients were involved in motor vehicle crashes, there was a broad spectrum of injuries. This included 26 with head injuries (half of whom had an Abbreviated Injury Score of 3 or more for this region). Nine patients had spinal fractures, 14 had lower extremity and/or pelvic fractures, 12 had thoracic injuries, and 7 had abdominal injuries. Despite these injuries, only 8 required blood transfusions during the 48-hour time span

of this study and only 9 underwent surgery (other than repair of lacerations) during this time.

### D-dimer Equivalent of XDP

The normal D-dimer equivalents of XDP as determined by ELISA ranged from 10 to 54 ng/mL (mean  $\pm$  SD =  $22.9 \pm 14.8$  ng/mL). The plasma D-dimer values measured in normals by LAT demonstrated immunoreactivities of less than 0.5  $\mu$ g/mL. The D-dimer values measured by both the LAT and ELISA showed a highly significant correlation in both normal and study samples (0.69,  $p = 0.0001$ , Fig. 1).

D-dimer values using both techniques were correlated with ISS in the patients and are shown for days 1-3 in Figures 2 (LAT) and 3 (ELISA). D-dimer by both techniques is elevated over normal human plasma (NHP) control values, with the greatest elevations being noted in the initial samples and a gradual return toward normal being noted on days 2 and 3, indicating that the trauma patient is hypercoagulable during this period of time. The data also demonstrate that there is no significant relationship between ISS and D-dimer values by either technique. Correlation coefficients and  $p$  values for LAT

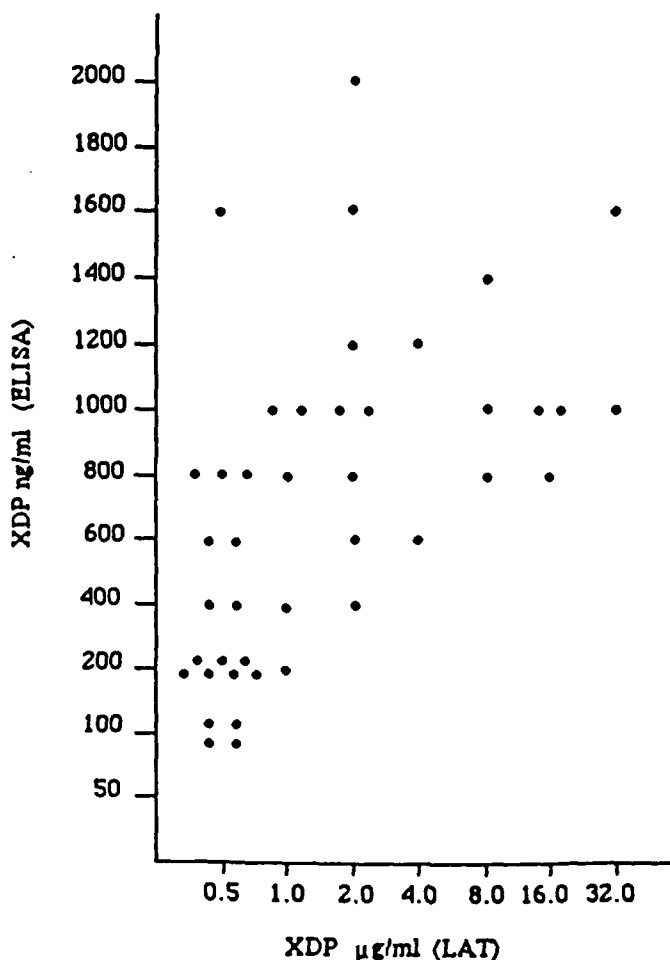


FIG. 1. Distribution of samples showing correlation of ELISA and LAT determination of D-dimer equivalents ( $r_s = 0.69$ ,  $p = 0.0001$ ).

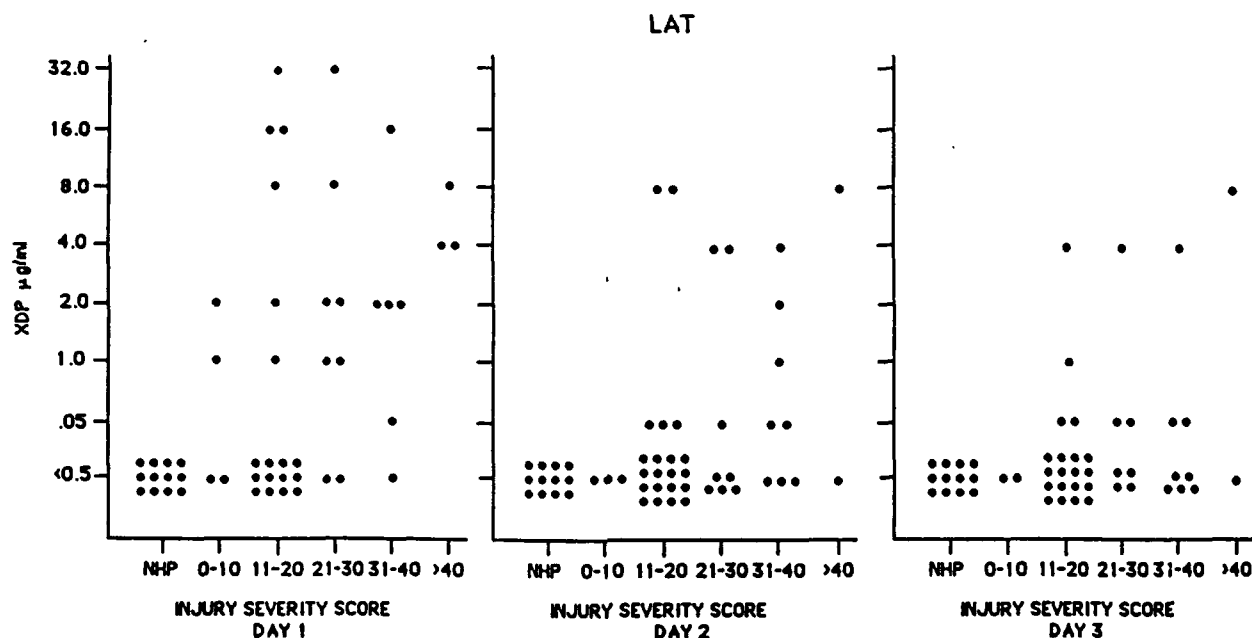


FIG. 2. Measurements of D-dimer immunoreactives ( $\mu\text{g/mL}$ ) as determined by latex agglutination in normal controls (NHP) and patients grouped by Injury Severity Score on days 1, 2, and 3. D-dimer levels increase initially (indicating hypercoagulability) and gradually return toward normal, but there is no correlation with ISS.

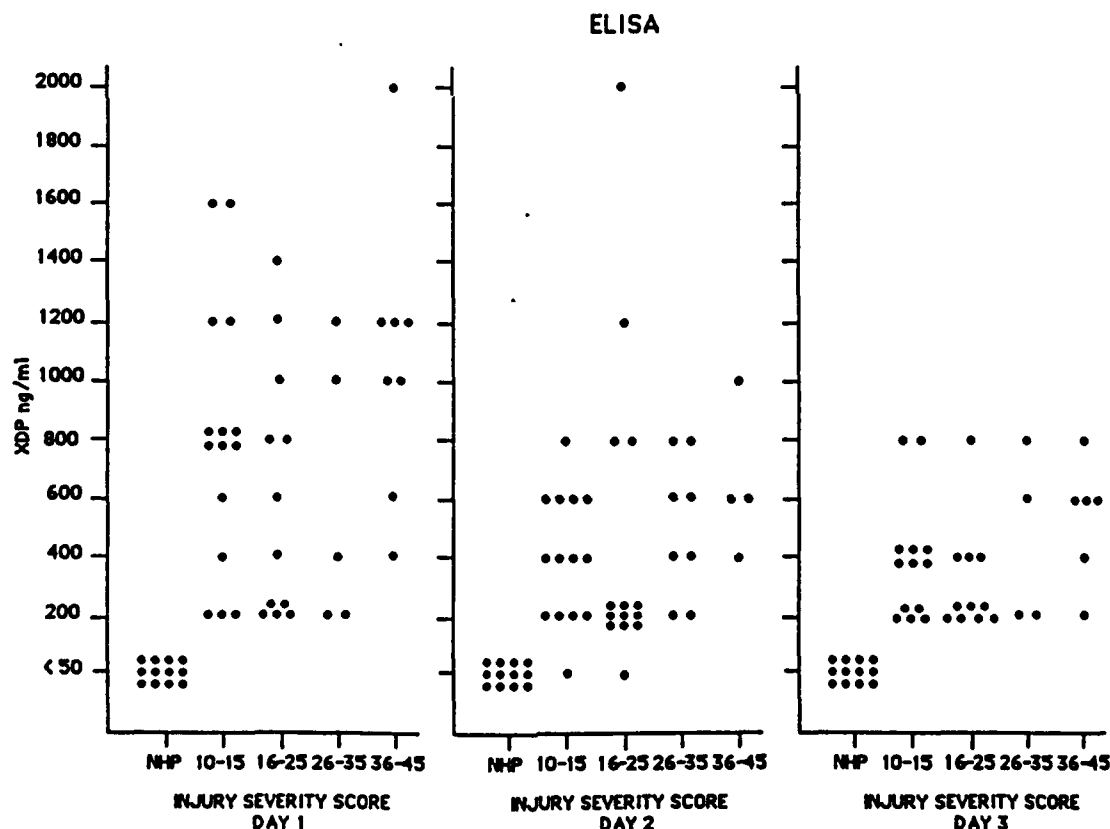


FIG. 3. Measurements of D-dimer ( $\text{ng/mL}$ ) as determined by ELISA in normal controls (NHP) and patients grouped by Injury Severity Score on days 1, 2, and 3. D-dimer behaves as it did in Figure 2.

vs. ELISA and ISS vs. ELISA are shown in Table 1 for days 1-3.

#### PA Activity by Fibrin Plate

Changes in PA activity as measured by fibrin plate are shown in Figure 4 for days 1-3 as means  $\pm$  SD and

compared with mean values for normal controls. These data show a suppression of PA activity (or suppression of fibrinolytic activity) when compared with normal subjects. The individual sample points are shown in Figure 5 for days 1-3. Four of the 42 patients showed increased PA activity upon admission when compared with normal

TABLE 1  
XDP data correlation

	Number	Correlation	p Value
Day 1			
Latex vs. ELISA	42	0.69	0.0001
ISS vs. ELISA	42	0.15	0.3424
Day 2			
Latex vs. ELISA	41	0.62	0.0001
ISS vs. ELISA	41	0.30	0.0544
Day 3			
Latex vs. ELISA	36	0.28	0.0978
ISS vs. ELISA	36	0.16	0.3313

ELISA and latex methods of measuring XDP levels: D1 and D2, significant correlation at  $p = 0.05$ .

## FIBRIN PLATE

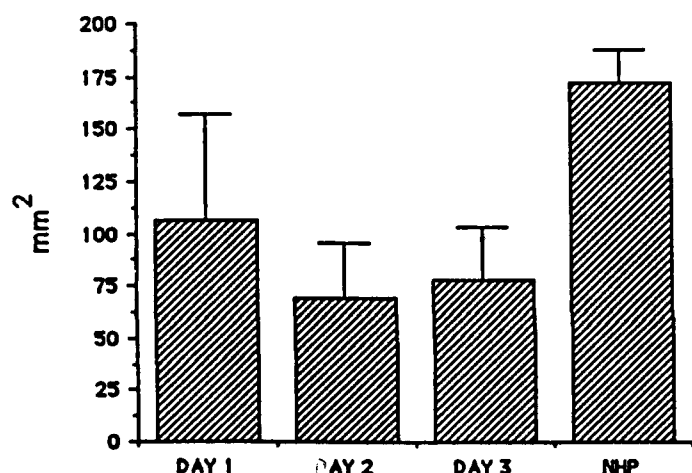


FIG. 4. Fibrinolysis (mean  $\pm$  SD) determined by fibrin plate demonstrates a decrease below normal (NHP) in trauma patients on days 1, 2, and 3.

controls. The majority of patients demonstrated suppressed PA activity. Thirty-two of the patients had fibrinolysis data drawn on all 3 days—four had the lowest level of PA activity on day 1, 14 on day 2, and 14 on day 3. This suppression of PA activity (suppression of fibrinolysis) was correlated with the elevation of D-dimer XDP (hypercoagulability) and was found to have a significant negative correlation (Table 2).

## AT-III and Other Tests of Coagulation

AT-III levels were measured in all patients entered into the study and compared with normal controls. These data are shown in Figure 6 as mean  $\pm$  SD and were not significantly different from normal controls on any of the days measured. As a general rule, clinical tests of coagulation (PT, APTT) did not differ significantly from normal, with only 4 patients demonstrating elevated PT and 8 patients having elevated APTT. These values also showed no correlation with ISS.

Comparison of D-dimer levels with specific injuries sustained, rather than ISS, demonstrated a greater elevation in D-dimer levels in patients with head and spinal cord injuries. Four patients with less severe injuries but

evidence of suppression of PA activity and elevated D-dimer levels developed symptoms of clinical thrombosis, including one patient who succumbed to a fatal pulmonary embolism.

## DISCUSSION

Hypercoagulability and suppression of fibrinolysis is a normal physiologic response after trauma, sepsis, burns, surgery, and shock and is probably teleologically a mechanism to minimize the danger of exsanguination. Garcia Frade et al. demonstrated suppression of fibrinolysis in 39 intensive care patients, only 12 of whom had sustained trauma.<sup>9</sup> Other studies have demonstrated hypercoagulability and variable results of fibrinolysis in patients who have sustained trauma.<sup>4,5</sup> Although the time course of these changes has not been completely described, it is clear that the hypercoagulable state occurs within hours of injury.

Normal hemostasis requires an exquisite balance between coagulation (to prevent exsanguination) and fibrinolysis (to prevent thrombosis).<sup>10,11</sup> This also requires coordination of a number of systems. Coagulation involves vessel constriction, platelet plugs, and the intrinsic and extrinsic systems of coagulation. The ultimate short-term goal of coagulation is the formation of a stable fibrin plug into which normal tissue can grow. The fibrin plug is formed by coagulation enzyme activation, which provides an amplified system to rapidly produce large amounts of fibrin. Activation of the fibrinolytic system is necessary simultaneously with the coagulation system to prevent overproduction of fibrin, which would result in pathologic thrombosis, and to remove fibrin once normal tissue and function have been restored.

The major fibrinolytic enzyme is plasmin, which is generated from plasminogen by both intrinsic and extrinsic activators.<sup>10</sup> The primary intrinsic activator is Hageman factor (XII), which generates plasmin at the same time that it activates the intrinsic coagulation system. However, the physiologic significance of intrinsic activation of the fibrinolytic system is uncertain and the molecular mechanisms involved have not been completely elucidated. Extrinsic plasminogen activators include tissue-type plasminogen activators and urokinase-type plasminogen activators. Other factors that enter into the balance between coagulation and fibrinolysis are inhibitors—both of plasmin (primarily alpha 2 antiplasmin) and of the plasminogen activators.

Plasmin is a proteolytic enzyme that degrades both fibrinogen and fibrin. Traditional measurements of fibrin degradation products (FDP) have been hampered by the fact that it does not distinguish between products resulting from the degradation of fibrinogen and fibrin.<sup>9</sup> Also, FDP determinations are usually done in serum rather than in plasma. In vitro, transformation of plasma into serum may either raise or lower FDP levels, depending on the preparation technique. Hence, testing of serum may lead to "spurious" measurements of FDP.

## FIBRINOLYSIS

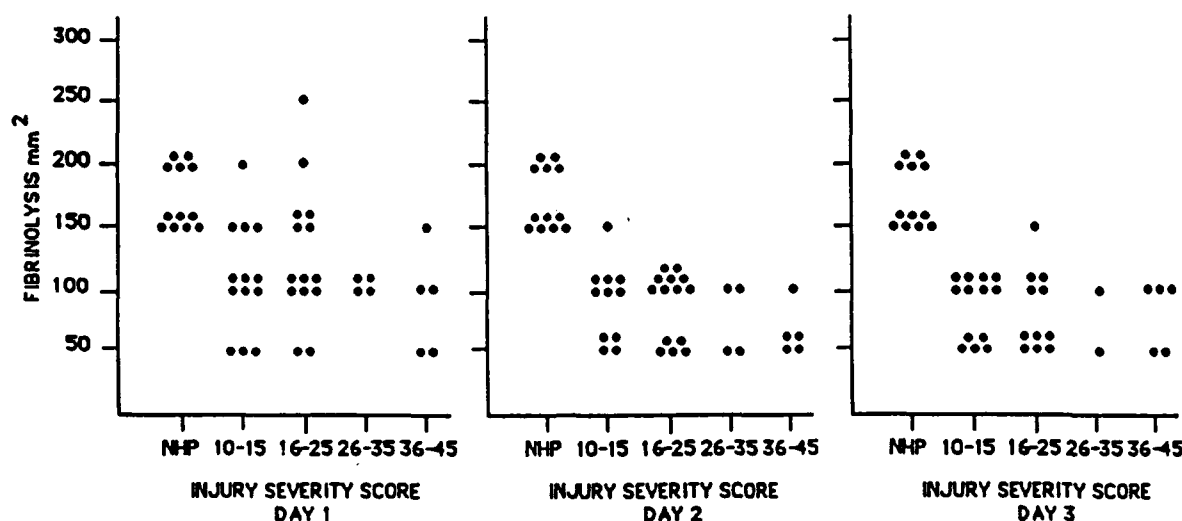


FIG. 5. Individual data points of fibrinolysis as determined by fibrin plate for days 1, 2, and 3 are grouped by Injury Severity Score. There is no correlation between fibrinolysis and ISS.

TABLE 2  
Fibrinolysis data correlation

	Number	Correlation	p Value
Fibrinolysis vs. corresp. XDP	31	-0.436	0.0071
Fibrinogen as % NHP vs. XDP	31	-0.339	0.0312
Fibrinolysis vs. max. XDP	31	-0.536	0.0010
Fibrinogen as % NHP vs. max. XDP	31	-0.450	0.0055

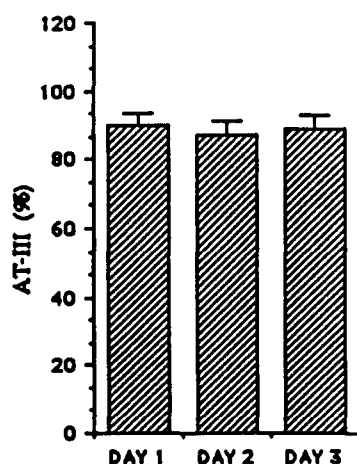


FIG. 6. Antithrombin III level in trauma patients (mean  $\pm$  SD), expressed as a percentage of normal on days 1, 2, and 3. There are no significant changes in antithrombin III levels.

Crosslinked fibrin is produced by the successive enzymatic actions of thrombin and factor XIII on fibrinogen, a circulating plasma protein. Thrombin releases fibrinopeptides A and B from fibrinogen to form fibrin monomers which polymerize to form a soluble fibrin polymer (non-crosslinked fibrin). Activated Factor XIII then mediates crosslinking between the gamma chains of this polymer, resulting in covalent bonding between the D moieties of adjacent fibrin monomers.<sup>12</sup> Cleavage of this stable fibrin plug by plasmin results in the production of

a heterogeneous group of XDPs with varying molecular weights. The smallest of the XDPs is D-dimer<sup>13</sup> (see Fig. 7). An assay for D-dimer is therefore indicative of lysis of stable, crosslinked fibrin.

Trauma patients often have problems with coagulation disorders that predispose them to both bleeding and thrombosis. The factors that play a role in these disorders have been difficult to ascertain because of an incomplete understanding of the mechanisms involved, variability of patients and injuries, and lack of specificity of tests

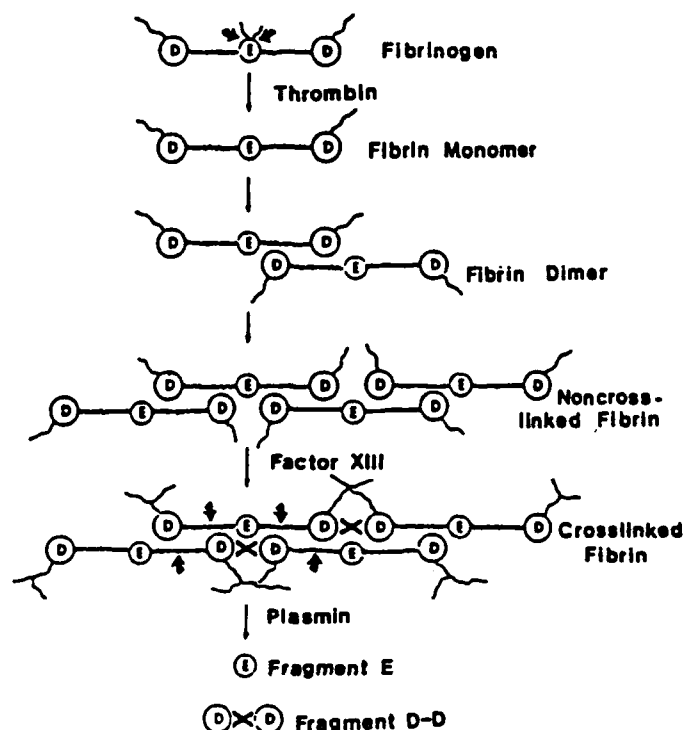


FIG. 7. Schematic representation of the formation of a stable cross-linked fibrin clot and its degradation by plasmin to form D-dimer crosslinked fibrin fragments.



used to evaluate them. Fibrinolysis has also been associated with complications in the trauma patient, such as adult respiratory distress syndrome (ARDS), through fibrin deposition or the effects of fibrin degradation products on the pulmonary microvasculature.<sup>14,15</sup> Finally, levels of fibronectin have been used as a prognostic factor or cause of mortality or ARDS.<sup>16</sup> Therefore, a better understanding of changes in coagulation/fibrinolysis in trauma patients may lead to fewer complications and improve survival.

Previous studies have demonstrated suppression of fibrinolysis and hypercoagulability occurring in trauma patients and in patients requiring intensive care.<sup>2,4,5</sup> Our data are consistent with these studies. An early decrease in coagulability and hyperfibrinolysis previously reported by Innes and Sevitt<sup>5</sup> was not seen in this study, perhaps because of differences in the time course involved or in measuring techniques. Borowiecki and Sharp reported large amounts of FDP in the serum of trauma patients but found no correlation of serum levels to the degree of injury.<sup>2</sup> One difference from our data is that they examined FDP, which looks at degradation products of both fibrin and fibrinogen, while we looked at D-dimer, which is specific for the degradation products of crosslinked fibrin.

The lack of correlation with ISSs or TSs is not too surprising because of the wide spectrum of injuries that these patients sustained. Looking at specific injuries revealed interesting trends, specifically the association of nervous system injury with increased D-dimer and suppressed PA activity. This coincides with data regarding hypercoagulable states in patients sustaining head<sup>17</sup> and spinal cord injury<sup>18</sup> and may warrant further and more specific investigation.

The other interesting finding was that several patients with relatively low degrees of injury showed increased D-dimer and suppressed PA activity and developed clinical evidence of thrombosis. This correlation was discovered retrospectively while trying to determine why these patients were markedly hypercoagulable. It was evident that there had been enough clinical suspicion of thrombosis by their primary services (who were unaware of the test results) that noninvasive flow studies and/or V/Q scans were obtained. One of the patients died suddenly and unexpectedly of a massive pulmonary embolism. Although we cannot draw firm conclusions from these anecdotal data, the relationship of increased D-dimer and pathologic thrombosis has been documented and deserves further investigation.<sup>19</sup>

The full dimension of the problem of pathologic thrombosis in trauma patients is not clear. However, it is probably greater than previously suspected.<sup>20</sup> Diagnosis of pathologic thrombosis using current techniques can be difficult, since definitive diagnosis requires invasive contrast studies. Noninvasive techniques, such as Dopplers, may be impossible to do because of extremity injuries and may not reflect the risk of pulmonary em-

bolism.<sup>20</sup> The measurement of D-dimer may be useful in identifying patients at risk of developing pathologic thrombosis. D-dimer provides a measure that is specific for XDPs and has been shown to correlate with thrombosis. The ELISA has been available but is difficult and time-consuming to run. The recent development of the LAT measure of D-dimer provides a technique that is more rapid and easier to conduct. This study demonstrates that the LAT and the ELISA give results that are highly correlated and the LAT shows promise in the management of trauma patients to evaluate the risk of thrombosis rapidly and repetitively.

### Acknowledgments

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## DISCUSSION

DR. GRACE S. ROZYCKI (Washington, DC): Annually, over 50,000 patients die of pulmonary embolus. This major health problem prompted the *Consensus Development Conference on Prevention of Venous Thrombosis and Pulmonary Embolism* in March 1986. Although high risk groups have been identified and recommendations made for prophylaxis, many issues remain unresolved. This work is an attempt to address one of those issues, i.e., the use of a rapid, reliable, highly sensitive screening test for thrombus formation.

In this manuscript, the authors prospectively examined the fibrinolytic system in 42 adult trauma patients. An increase in the D-dimer crosslinked fibrin fragment, the smallest and most characteristic of fibrin breakdown products, was found to be indicative of a hypercoagulable state, especially in patients with nervous system injuries. Their data are supported by the fact that two different assay methods, LAT and ELISA, showed a highly significant correlation in both normals and study samples. However, their patient population of 42 is too small to rule out a correlation between ISS and TS. In addition, there is no description of case mix, length of stay in ICU, or complications.

I have several questions for the authors: (1) Although some studies support that elevated D-dimer levels may be of diagnostic value in suspected pulmonary embolus, there are other causes of elevated D-dimer levels, such as large hematomas, acute MI, and malignancy. Did you characterize your patient population specifically looking for premorbid factors that could

influence D-dimer levels? (2) Is the test specific enough to distinguish between a coexisting large perinephric hematoma and a small vessel thrombus, each projecting different risks for deep vein thrombosis? (3) Did any of the patients undergo massive transfusion during resuscitation and if so, how would you suspect this would alter your results? (4) Of those "several patients" who had V/Q scans, was there a D-dimer level that correlated with positivity? (5) Based on your research, do you postulate that there may be differences in individual fibrinolytic capacity of tissue types explaining the variances in D-dimer levels?

I enjoyed reading this paper very much and congratulate the authors for an important piece of work on a timely subject. I would like to thank the Association for the opportunity to review this paper and the privilege of the floor.

DR. BLAINE ENDERSON (Closing): Thank you, Dr. Rozycki, for your comments and questions. Basically in answer to all your questions, as you pointed out our sample was fairly small, and it was a diverse population. We were not able to specifically characterize or determine the specificity of the test. We think we have a test that can be done rapidly and deserves further investigation. For right now, we have not been able to sort out the first couple of questions you asked.

As far as massive transfusion, a couple of the patients did undergo massive transfusion, and we did not see any specific changes of D-dimer levels associated with the massive transfusion. I would expect that with more numbers, we would be able to tell more about that. As far as a correlation of the D-dimer level with positive tests of thrombosis, we do not have a specific D-dimer level that we have correlated, since we found this in a retrospective fashion in patients who had very high D-dimer levels and very low levels of associated injuries. It is something we are looking at further, and we are conducting a prospective study and looking at the other factors that may be involved in this system.

I would like to thank the Association for the privilege of the floor.

RADIOIMMUNOASSAY OF GUINEA PIG FIBRINOPEPTIDE A AND CROSS-REACTIVITY WITH FIBRINOGEN AND FIBRIN(OGEN) DEGRADATION PRODUCTS

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University of Tennessee Medical Center\* and  
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TN, U.S.A.

Pichinde virus infection of strain 13 guinea pigs is a model for arenaviral infections in humans. Infected animals show decreased activity of multiple coagulation factors, high serum levels of fibrin(ogen) degradation products (FDP), impaired platelet function and thrombocytopenia.

A radioimmunoassay (RIA) of guinea pig fibrinopeptide A (gFPA) has been developed to measure the degree of thrombin generation and action in vivo of infected animals. gFPA was synthesized via the solid-phase method, and conjugated to bovine serum albumin (BSA) by two different methods of chemical coupling, i.e., with glutaraldehyde or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl. A double antibody RIA was established employing goat anti-rabbit IgG to precipitate the primary complex composed of  $^{125}\text{I}$ -5-tyrosyl-gFPA and rabbit anti-gFPA-BSA. The working range for gFPA was between 1 ng/ml and 1  $\mu\text{g/ml}$ . Guinea pig fibrinogen inhibited the primary antigen-antibody reaction from 1 to 4  $\mu\text{g/ml}$  or 2.9-12 pmole/ml. We have also determined the extent of cross-reactions exhibited with FDP which are expected to be present in arenaviral infection. Rabbit anti-gFPA-BSA showed an extensive cross-reactivity with fragment E, but not with D. The gFPA-RIA will be validated by determining the quantity of gFPA released from thrombin-degraded guinea pig fibrinogen. After validation, plasma gFPA immunoreactivities will be determined in strain 13 guinea pigs infected with Pichinde virus.

Sample Abstract

DIFFERENTIAL BINDING OF RECOMBINANT WILD TYPE AND VARIANT t-PA TO HUMAN ENDOTHELIAL CELLS

E. S. Barnathan, D. B. Cines, K. Barone, A. Kuo, G. R. Larsen

University of Pennsylvania, Philadelphia, PA, and  
Genetics Institute, Cambridge, MA, USA

Human endothelial cells in culture synthesize and secrete t-PA. We found that cultured human umbilical vein endothelial cells (HUVEC) also bind added radiolabeled recombinant wild type t-PA

Date: March 8, 1990

Signature: James P. Chen

Title of Abstract:

Radioimmunoassay of Guinea

Pig Fibrinopeptide A and

Cross-Reactivity with

Fibrinogen and Fibrin(ogen)

Degradation Products

DR. N. U. BANG

Lilly Clinic

Wishard Memorial Hospital

1001 West 10th Street

Indianapolis, Indiana 46202

U.S.A.

Tenth International  
Congress On  
**Fibrinolysis**

Cover:  
Three-dimensional structure of Kringle 2 of t-PA.  
Courtesy: Drs. Jan McDonagh and Eric Fend

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RADIOIMMUNOASSAY OF GUINEA PIG FIBRINOPEPTIDE A: A MEANS  
TO DEDUCE THE PATHOGENESIS OF PICHINDE VIRUS INFECTION  
J.P. Chen, L.C. Marsh and E.C. Schroeder. Univ. Tenn. Med.  
Ctr. and Col. Vet. Med., Knoxville, TN 37920

Pichinde virus infection of strain 13 guinea pigs is  
a model for arenaviral infections in humans. Infected  
animals experience reduced levels of coagulation factors  
caused by either consumption coagulopathy or impaired  
synthesis due to liver injury.

A radioimmunoassay (RIA) of guinea pig fibrinopeptide  
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pig fibrinogen inhibited the primary antigen-antibody  
reaction from 1 to 4 µg/ml. Both fragments D and E showed  
an extensive cross-reactivity with gFPA. Fibrin(ogen)  
degradation products are known to be present in arenaviral  
infection. Plasma gFPA immunoreactivities were determined  
in outbred guinea pigs after removing cross-reactive material  
by heating with polyethylene glycol and dialysis (Ave.  
values 77.1 ng/ml). The elevation of gFPA immunoreactivities  
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